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CONTENTS

Mitsunori WADA: Isolation of Citrullin, δ-Carbamido-Ornithin, from the Tryptic Digestion Products of Casein. ... 45
Mitsunori WADA: Über Prolysin, α-Amino-ε-hydantoincapronsäure, ein neues Abbauprodukt des Eiweisses. I. ... 49
Kotaro NISHIDA: On the so-called "Imo-shōchū" the Sweet potato spirit by Old Method. ... 54
Tomotsune TAIRA: Über Säuren und Alkohols im hochsiedenden Anteil des Fuselöls. ... 57
Yoshikazu SAHASHI: Nutritive Value of Sperm Whale Oil and Finback Whale Oil. ... 60
Midzuho SUMI: On the Ergosterin Content of Various Edible Mushrooms in Japan. ... 69
S. IZUME, Y. YOSHIMURA and Y. HIDAKA: Studies on Experimental Rickets, IV.—On the Occurrence of Ergosterol in Shoyû Oil. ... 73
M. TOKI and T. MIYOSHI: Studies on Blood Pressure Decreasing Substances, Part 2.—Yeast nucleic acid. ... 79
Sogo TETSUMOTO: Sterilising Action of Acids, III Report.—Sterilising action of saturated monobasic fatty acid on putrifactive bacteria, Bac. typhosus and V. Cholerae (The Second Report). ... 82
Shûiku SASAKI: Studies on the Germination of Seed, Part III.—Organic Bases of the Seeds, and Seedlings of Soy-Bean Germinated in a Dark Room. ... 87
Masami OKU: On the Natural Pigments of Raw Silk Fibre of the Domestic Coccon, Part V.—Detection of Violaxanthin in the yellow Cocoon. ... 91

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The articles to be appeared in the Bulletin must be concise, supplied with experimental methods and data and understandable, without specially referring to the Japanese texts. It ought, however, not exceed four printed pages as a rule. Any longer articles may be accepted according to the decision of the Council, with or without charge for exceeding pages.

Journal of the Agr. Chem. Soc. of Japan will be published in Japanese as formerly. Those desiring the detailed information of the articles appeared in the Bulletin may look for in the Journal of the same Number or the same Volume.

Editor : Kintaro OHSHIMA.
Associate Editors : Kakuji GOTŌ and Yoshikazu SAHASHI.

Isolation of Citrullin, δ-Carbamido-Ornithin, from the Tryptic Digestion Products of Casein.

By

Mitsunori WADA.

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(Received February 13, 1933)

Two years ago,⁽¹⁾ the author has isolated a new amino acid, Citrullin, from the pressed juice of water melon, Citrullus vulgaris, Schrad. and proved it to be δ-carbamido-ornithin, represented by the following formula:

$$NH_2\text{-}CO\text{-}NH \cdot CH_2CH_2CH_2CH(NH_2)COOH.$$

This formula was further confirmed by the synthesis.

Since then, the author has tried to isolate this substance from the hydrolytic products of proteins and studied its behaviour toward acids and alkalies and observed that it is converted to prolin when heated with concentrated acids, while by the action of dilute alkali it is turned into ornithin, carbamino group being splitting off. These facts indicate that citrullin can not exist as such in the acid- or alkaline hydrolytic products, so the author investigated the enzymatic digestion products of proteins. In the first experiment when casein was digested with trypsin in a 0.2% Na-carbonate solution, citrullin could not be isolated and instead of it, ornithin was obtained in fairly good yield. In the next experiment, however, when the digestion was carried out in a neutral solution for 30 days, citrullin was really isolated as copper salt, from which the free acid was regenerated and proved to be identical with the natural product, obtained from water melon. The isolation process was nevertheless very tedious and the yield was far smaller than it was expected. In the third experiment, the method was much simplified and the yield was somewhat better. Still better result is now expected by the tryptic digestion in dilute ammoniacal solution. The details of which will be reported later on.

Experimental

1. Tryptic digestion of casein in a 0.2% sodium carbonate solution.

470 g. commercial casein were rubbed in a mortar with water, neutralised with Na_2CO_3, brought in a large flask, diluted with water to 2 liters and so much anhydrous sodium carbonate was added until it reaches 0.2% of the liquid and after adding 2.5 g. trypsin (Merck) and 10 ccm. toluol, the flask was kept at 37° with frequent shaking. From time to time the reaction was tested with congo paper and so much sodium carbonate was added until the paper turned red. After 30 days when the solution became yellowish

brown and white crystalline cluster deposited at the bottom, it was filtered, neutralized with sulphuric acid, evaporated to 500 ccm., acidified with sulphuric acid and treated with phosphotungstic acid. The precipitate formed thereby was collected by suction, dissolved in 50% acetone, decomposed with baryta in usual way and the filtrate containing free bases was treated with 30% silver nitrate and baryta until no more precipitate was formed, filtered off and the filtrate i.e. the lysin fraction was freed from silver and baryta by sulphuric acid and hydrogen sulphide respectively, evaporated to 400 ccm., acidified with sulphuric acid and once more precipitated with phosphotungstic acid. The free bases regenerated from this precipitate were converted into picrates, which were revealed to be the mixture of ornithin and lysin. By treating the above picrates with methyl alcohol, ornithin picrate was dissolved leaving lysin picrate as insoluble residue. The methyl alcoholic extract was now evaporated in vacuum, again treated with methyl alcohol to remove the little insoluble residue. After repeating this operation five times 49.9 g. ornithin picrate (ca. 3% of casein used) were obtained. It was converted into copper salt and analysed:

7.198 mg. subst. 1.041 ccm. N_2(757.6 m.m. 14°) = 16.88% N
Calculated for ornithin copper = 17.19% N

2. Tryptic digestion of casein in neutral solution.

500 g. commercial casein were rubbed in a mortar with water, adding sodium carbonate until neutral reaction was attained, the thick paste thus obtaind was brought in a large flask, diluted with 6 liters water and after adding 5 g. trypsin (Merck) and 10 ccm. toluol, the flask was kept at 37°, shaking from time to time. The acidity developed during the digestion was carefully neutralized with sodium carbonate. After two weeks the reaction became constant, so that the neutralization was no more necessary and greyish white crystalline cluster, consisting chiefly of tyrosin began to deposit at the bottom. After 30 days it was filtered and the yellowish brown filtrate was concentrated in vacuum to a small volume and treated with five times of its volume of methyl alcohol. The precipitate formed thereby was collected after 24 hours, dissolved in water and fractionally precipitated with silver nitrate and baryta. The last fraction obtained by adding an excess of baryta, consisted of a reddish brown precipitate giving the typical reaction of citrullin. It was treated with sulphuric acid and then with hydrogen sulphide to remove the baryta and silver completely, and after expelling off the hydrogen sulphide, the citrullin was isolated as the copper salt. The yield was 1.05 g. The free citrullin regenerated from the copper salt forms colourless long thin prisms, melting at 226°. In every respect it was identical with the natural product. It was dried at 100° and analysed:

5.648 mg. subst. 1.225 ccm. N_2(757.5 m.m. 28°)=23.64% N
3.786 mg. subst. 5.661 mg. CO_2=40.78% C 2.595 mg. H_2O=7.62% H

Found	C%	40.78	H%	7.62	N%	23.64
Calc. for $C_6H_{13}N_3O_3$		41.11		7.48		23.99

The free base was again converted into copper salt and analysed:

5.761 mg. subst. 1.008 ccm. N_2(757,6 m.m. 14.5°)=20.36% N
4.428 mg. subst. 5.533 mg. CO_2=34.07% C 2,339 mg. H_2O=5.86% H.

Found	C%	34.07	H%	5.86	N%	20.36
Calc. for $(C_6H_{12}N_3O_3)_2Cu$		34.79		5.84		20,31

When citrullin was precipitated with silver nitrate and baryta as described in the above experiment and the precipitate was directly boiled with 5 ccm. hydrogen iodide for 8 hours, citrullin was converted into prolin. For the isolation of the latter, the boiled mixture was diluted with water, filtered and evaporated to expell off the HI, boiled with copper hydroxide, filtered, evaporated and treated with methyl alcohol. The copper salt of prolin obtained in this way was purified by recrystallisation, dried at 100° and analysed:

5.184 mg. subst. 0.438 ccm. N_2(759.3 m.m. 17°)=9.75% N
Calc. for prolin copper =9.87% N

3. Formation of prolin from citrullin.

The formation of prolin from citrullin by the action of strong acids is demonstrated in the following experiment:

0.5 g. citrullin, isolated from water melon was boiled with three times of conc. HCl for 8 hours under reflux cooler. After distilling off the hydrochloric acid in vacuum, the residue was dissolved in a little water and boiled with $Cu(OH)_2$. The filtrate therefrom was evaporated and treated with ethyl alcohol, the soluble cupric chloride was filtered off, and the precipitate was washed with ethyl alcohol. It was easily soluble in water, but almost insoluble in methyl alcohol. In this way, the copper salt of prolin was obtained. After recrystallization, it was dried at 100° and analysed:

3.030 mg. subst. 0.270 ccm. N_2(759,3 m.m. 26°)=9.89% N
Calc. for prolin copper $(C_5H_8NO_2)_2Cu$ =9.87% N

From the copper salt, free prolin was prepared. It was dissolved in ethyl alcohol and precipitated with ether, the precipitate thus obtained was again extracted with butyl alcohol, once more recrystallized from ethyl alcoholic solution by adding ether, dried at 100° and analysed:

5.961 mg. subst. 0.659 ccm. N_2(754.8 m.m. 25°)=11.90% N
Calc. for prolin $C_5H_9NO_2$ =12.18% N

Heated in a capillary it begins to melt at 200° and decomposes at 220°: By fusion the characteristic odour of pyrrolidin is developed.

The author has further observed that ornithin is very resistant toward strong acids. Even by prolonged heating with conc. hydrochloric acid only a portion of it is converted into prolin. Further, the fact that ornithin is never formed by the acid hydrolysis of proteins indicates that it is not the primary decomposition products of proteins, so it is self-evident that prolin can not be derived from ornithin.

As prolin is rather easily formed from citrulin by the action of acid it is more probable that during the acid hydrolysis, at least a portion of prolin is derived from citrullin, though it may also be formed as the primary product.

Ornithin may be formed secondarily either from arginin or from citrullin during the alkaline hydrolysis or during the tryptic digestion of proteins. The author has further estimated the distribution of different amino acids according to the method of van Slyke in the acid and alkali hydrolytic products of proteins and found that the nonamino-N, i.e. the prolin fraction is always higher in the acid hydrolysis. This might be due to the formation of prolin, at least partly, from citrullin.

D. Ackermann⁽²⁾ has recently reported on the formation of citrullin from arginin by the putrifying process. In the experiment of the present auther however no bacterial growth was observed during the tryptic digestion of casein, so it is evident that microorganisms has no casual relation with the formation of citrullin from proteins, but the question whether citrullin might be formed from arginin by the action of trypsin, remains to be settled. For such a reason the author carried out the following experiment:

2.752 g. arginin carbonate were dissolved in 27 ccm. water to which 0.1 g. trypsin (Merck) was added besides a little chloroform and toluol. After standing for 6 weeks at 37°, the solution was filtered and treated with 5.5 g. flavianic acid and a few drops of conc. HCl and after warming for a short time, it was left for 24 hours when nice plate crystals of arginin flavianate separated out, which were collected by suction, washed with ethyl alcohol and dried in a vacuum desiccater. In this way 5.95 g. arginin flavianate, corresponding to 2.752 g. arginin carbonate were recoverd. As no citrullin could be detected in this case, it is evident that trypsin had no action upon arginin.

Literature

(1) M. Wada: P. I. A. **6**, 15~17, 1930., Biochem. Zeit., **224**, 420~429, 1930.

(2) D. Ackermann: Zeit. physiol. Chem., **203**, 66~69, 1932.

Über Prolysin, α-Amino-ε-hydantoincapronsäure, ein neues Abbauprodukt des Eiweisses. I.

Von

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(Eingegangen am Februar 13. 1933)

Vor Kurzem, als der Verfasser sich mit der Isolierung des Citrullins aus tryptischen Verdauungsprodukten des Caseins beschäftigte, beobachtete er, dass der Verdauungssaft ausser Citrullin noch eine Substanz enthält, welche die für Harnstoffgruppen charakteristische Schiffsche sowie P. Ehrlichsche Reaktion gibt. Diese Substanz ist gegen Säuren beständiger als Citrullin und existiert noch nach dem Zerstören des Citrullins durch verdünnte Salzsäure in die Flüssigkeit. Sie entsteht auch bei vorsichtiger Verdauung des Eiweisses durch verd. Säuren. Durch längeres Erhitzen mit konz. Säuren order Alkalien wird sie aber vollständig zerstört. Am besten kann man eine alkoholische Salzsäurelösung für die Verdauung anwenden.

Seitdem hat der Verfasser sich bemüht, diese Substanz zu isolieren. Wenn die Verdauungsflüssigkeit, die obengenannten Reaktionen gibt, bis zu schwach saurer Reaktion neutralisiert, mit Tierkohle entfärbt und mit einer verd. alkoholischen Lösung von Xanthydrol versetzt wird, so kann man diese Substanz als Xanthylverbindung in farblosen Prismen bekommen. Ferner, wenn die neutralisierte Verdauungsflüssigkeit nach starkem Einengen im Vakuum mit fünffacher Menge Methylalkohol versetzt wird, so entsteht ein Niederschlag, der starke Schiffsche Reaktion gibt. Wird nun dieser Niederschlag in Wasser gelöst und mit Silbernitrat und Baryt fraktioniert gefällt, so erhält man diese Substanz als Silbersalz, welches in üblicher Weise in kristallinisches Kupfersalz verwandelt und gereinigt wird.

Die aus reinem Kupfersalz regenerierte freie Substanz bildet ein hygroskopisches, weisses, kristallinisches Pulver, das löslich in Wasser, unlöslich in Alkohol und Aether ist. Es gibt die Schiffsche, P. Ehrlichsche Jaffesche sowie die Biuret und Triketohydrinden Reaktionen; es bildet hellblaues Kupfersalz wie gewöhnliche Aminosäuren und auch kristallinisches Pikrat. Gegen Xanthydrol verhält es sich wie Harnstoff und bildet kristallinische Monoxanthylverbindung.

Die Analyse der freien Substanz stimmt mit der Formel $C_8H_{13}N_3O_4$ überein.

Beim Kochen mit konz. Säuren oder Alkalien wird sie glatt in Lysin gespalten. Wahrscheinlich bietet sie eine Vorstufe des Lysins in Eiweissmolekül dar. So hat der Verfasser den Namen *"Prolysin"* für diese Substanz vorgeschlagen.

Durch Reduktion und Hydrolyse des Prolysins mit Jodwasserstoff bildet sie α–Monoaminopimelinsäure, die weiter in Pimelinsäure gespalten wird.

Durch Einwirkung von Bariumhydroxyd bei Zimmertemperatur wird zuerst α–Uramino–α'-aminopimelinsäure gebildet, welche weiter in Diaminopimelinsäure verwandelt wird.

Schwefelwasserstoff und Bariumcarbonat spalten sie in α–Uramino–α'–aminopimelinsäure und weiter in Harnstoff und α–Aminopimelinsäure.

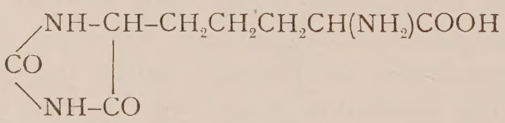
Durch Salpetersäure bei gewöhnlicher Temperatur oxydiert, wird Parabansäure gebildet.

Mit Harnstoff kondensiert, bildet sie Pentamethylendihydantoin, die durch Erhitzen mit Bariumhydroxyd in Cadaverin verwandelt wird.

α–Aminopimelinsäure liefert in analoger Weise mit Harnstoff ε–Hydantoincapronsäure, die beim Kochen mit Bariumhydroxyd wieder in ε–Aminocapronsäure gespalten wird.

Durch Jodierung des Prolysins werden zwei Wasserstoffatome der Imidoradikales substituiert.

Aus den oben erwähnten Beobachtungen hat der Verfasser dem Prolysin die folgende Formel zugeschrieben:



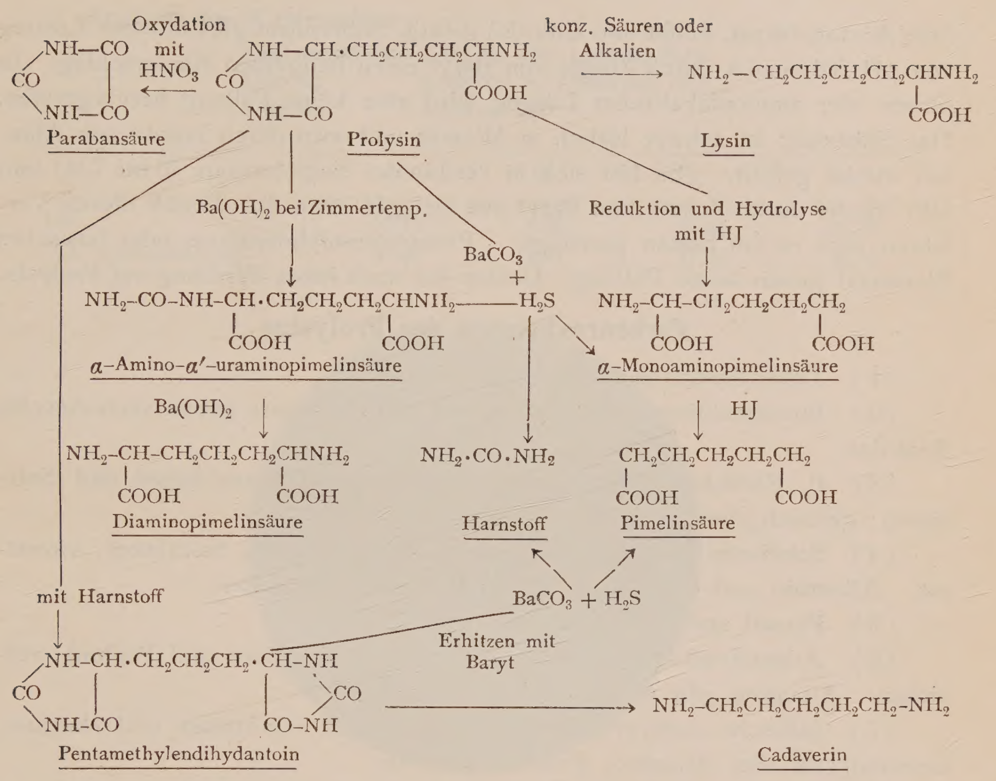
$$\begin{array}{l} \diagup NH-CH-CH_2CH_2CH_2CH(NH_2)COOH \\ CO \qquad | \\ \diagdown NH-CO \end{array}$$

Prolysin: α–Amino-ε–hydantoincapronsäure.

Umwandlung und Spaltungsweise des Prolysins werden in der folgender Seite schematisch dargestellt.

Isolierung des Prolysins

900 g Gelatine wurden mit 900 ccm salzsäuregesättigtem, 60%igem Alkohol 8 Stunden gekocht (die Temperatur der Flüssigkeit war etwa 90°). Das Hydrolysat zeigte starke Biuretreaktion sowie die Schiffsche Reaktion (rotviolette Färbung mit Furfurol, Aceton und konz. Salzsäure). Es wurde mit 300 ccm 50%iger alkoholischer Natronlauge neutralisiert, eingedampft, filtriert und mit 50%igem Alkohol gewaschen. Das Filtrat wurde dann im Vakuum zum Sirup eingedampft und mit fünffacher Menge Methylalkohol versetzt, wodurch ein Niederschlag entstand, welcher starke Schiffsche sowie P. Ehrlichsche Reaktion gab. Der Niederschlag wurde in Wasser gelöst und mit Silbernitrat und Baryt fraktioniert gefällt. Da die in saurer Lösung gebildete gelbbraune Fällung kein Prolysin enthielt, wurde sie abfiltriert und zum Filtrat soviel Baryt zugegeben bis es pH 5.8 erreichte. Das Prolysin wurde dadurch fast



vollständig als hellgelber amorpher Niederschlag gefällt. Nach einem Tag wurde es abfiltriert, sukzessiv mit wenig Wasser, Methylalkohol und Aether gewaschen und im vakuum über Schwefelsäure getrocknet. Die Ausbeute des Silbersalzes betrug etwa 12 g. Um freies Prolysin darzustellen, wurde das Silbersalz in 50%igem Alkohol suspendiert, durch Schwefelsäure und Schwefelwasserstoff von Baryt bezw. Silber befreit, eingedampft und mit Methyalkohol und Aether behandelt. In der Weise erhielt man das Prolysin als weisses, kristallinisches Pulver. Es bildet hellblaues kristallinisches Kupfersalz vom Schmelzp. 260°, welches in Wasser sehr leicht, in Methyl- und Aethylalkohol aber unlöslich ist. Das Pikrat des Prolysins kristallisiert in gelben Plättchen, schmilzt bei 122°. Es ist in Wasser leicht, in Methylalkohol und Aether unlöslich. Das reine freie Prolysin, regeneriert aus reinem Kupfersalze, ist ein weisses, kristallinisches Pulver, löslich in Wasser, unlöslich in Methyl- Aethyl- Butylalkohol, Aceton, Aether usw. Im Kapillarrohr erhitzt, beginnt es bei 209° sich zu färben und zersetzt es sich bei 222°.

Die wässerige Lösung reagiert neutral gegen Lakmus. Aus konzentrierter Lösung wird es durch Phosphorwolframsäure gefällt, nicht aber aus verdünnter Lösung. Aus alkalischer Lösung wird es durch Quecksilbersalze, (wie Acetat, Nitrat, Sulfat und Chlorid) gefällt. Silbernitrat gibt in einer Lösung von pH 5.6 bis 6.8 durch Zusatz von Baryt einen hellgelben Niederschlag. In saurer oder ammoniakalischer Lösung wird aber keine Fällung hervorgerufen. Das Silbersalz ist schwer löslich in Wasser und wird durch Zusatz von Alkohol wieder gefällt. Es löst sich in verdünnter Salpetersäure (6 bis 7%) und fällt wieder durch Zusatz von Baryt aus (bei pH etwa 6). Durch dieses Verfahren wird es am besten gereinigt. Phosphormolybdänsäure oder basisches Bleiacetat geben keine Fällung. Urease hat auch keine Wirkung auf Prolysin.

Farbenreaktionen des Prolysins

(1) Triketohydrindenreaktion: violett.

(2) Biuretreaktion: stark. Allantoin und Harnsäure geben auch dieselbe Reaktion.

(3) P. Ehrlichsche Reaktion (p-Dimethylaminobenzaldehyd und Salzsäure): gelblichgrün.

(4) Schiffsche Reaktion (Furfurol, Aceton und konz. Salzsäure): violettrot. Allantoin und Citrullin geben auch dieselbe Reaktion.

(5) Phenol und Natriumhypochlorit: blau.

(6) Adamkiewiczsche Reaktion (konz. Schwefelsäure und Pepton): rotviolett. Allantoin gibt wieder dieselbe Reaktion.

(7) Jaffesche Anhydridreaktion (Erhitzen mit Pikrinsäure und Natriumcarbonat): rot. Bei Allantoin dieselbe Reaktion.

(8) 1% Pyrogallol in konz. Schwefelsäure: schön rotviolett. Allantoin: gelblichgrün.

(9) 0,1% Indollösung und konz. Schwefelsäure: rot.

(10) 0,1% Skatollösung und konz. Schwefelsäure: gelblichrot.

(11) Paulysche Diazoreaktion: orange.

(12) Fehlingsche Lösung: beim längeren Erhitzen reduziert.

(13) Ferriferricyanidreagens: grünlichblau.

(14) Pyrrolreaktion: stark.

(15) Ferrichlorid: rote Fällung.

(16) Die folgenden Reaktionen fallen negative aus: Millon, Sakaguchi, Murexid, Folin, Mörner und Liebermannsche Nitrosoprobe.

Analyse des freien Prolysins:

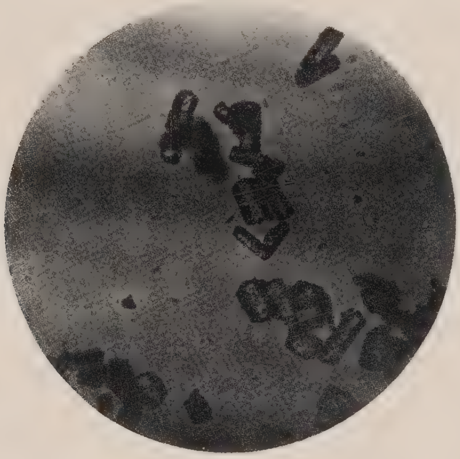
4,946 mg Substanz 8,043 mg CO_2, 2,955 mg H_2O,
6,723 mg Substanz 1,108 ccm N_2 (758,1 mm 17°)

	C	H	N
Gef.	44,34	6,63	19,33
Ber. $C_8H_{13}N_3O_4$	44,62	6,09	19,53

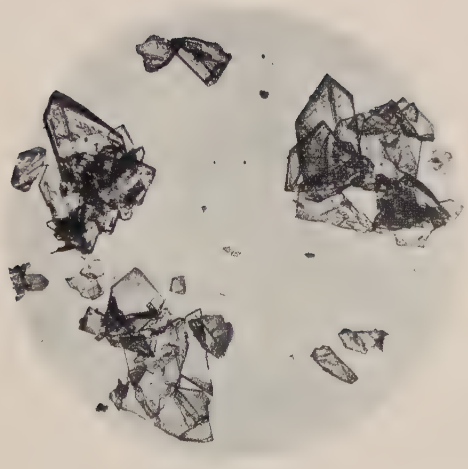
Analyse des Kupfersalzes:

3,383 mg Substanz 4,861 mg CO_2, 1,523 mg H_2O,
3,505 mg 〃 0,508 ccm N_2 (759,8 mm 16°),
3,383 mg 〃 0,554 mg CuO

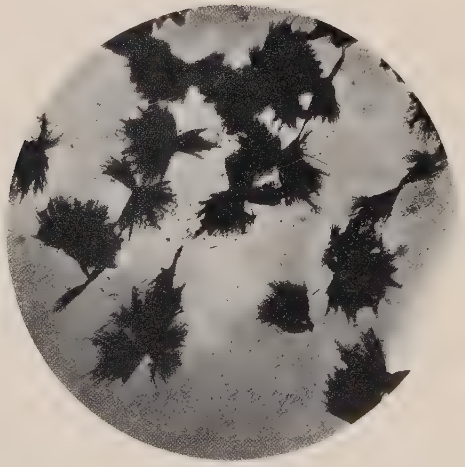
	C	H	N	Cu
Gef.	39,19	5,00	17,11	12,97
Ber. $(C_8H_{12}N_3O_4)_2Cu$	39,03	4,91	17,08	12,92



Monoxanthylprolysin 1:265



Prolysinpikrat 1:51



α-Monoaminopimelinsäure 1:165
(Kupfersalz)

On the so-called "Imo-shōchû" the Sweet potato spirit by Old Method.

By

Kotaro NISHIDA

(Kagoshima Agricultural College, Kagoshima, Japan.)

(Received February 28, 1933.)

The so-called "Imo-Shōchû" is a kind of spirit obtained from "Imo" (Sweet potato) and "koji" by old method. It is a special product of two prefectures—Kagoshima and Miyazaki—in our country. The annual production of this spirit in these districts amounts to about 100,000 hectolitres.

As its raw materials there are used (1) steamed sweet potato, (2) 'Awamori koji" or black koji, a steamed foreigen rice upon which *Aspergillus Awamori* has been developed, and (3) water.

The spirit mash is prepared by mixing these raw materials in following proportion.

	In the 1st mixing	In the 2nd mixing	Total	Riped mash
Sweet potato	——	450 kg	450 kg	785 litres
"Awamori koji" rice	130 litres	——	130 litres	
water	145 litres	247 litres	392 litres	

In a large earthen pot, "Awamori koji" prepared from 130 litres of foreign rice, is allowed to stand in 145 litres of water for about a week. Then the second mixing materials is added to the first mixture, and after about 10 days, the mash is distilled by very simple distillator. The yield of the sweet potato spirit, containing 40% alcohol by volume, is about 244 litres from 785 litres of riped mash.

Experimental Results.

I. Chemical Composition of "Awamori koji" and its Extract.

The analytical results of "Awamori koji" and its saccharified extract (Balling=10°, PH=3.11), are shown in the following table:

	In 100 g of "Awamori koji" (g)	In 100 c.c. of koji extract (g)
Moisture	21.79	90.31
Solid matter	78.21	9.69
Total N	1.1062	0.1274
Protein N	0.9544	0.0038

Non-protein N	0.1518	0.1236
Ether extract	0.2916	——
Alcohol extract (free from ether extract)	1.9883	——
Reducing sugar (as glucose)	1.8537	7.1076
Starch and Dextrin	62.13	——
Total carbohydrate (as glucose)	——	8.9223
Dextrin	——	1.6332
Total acid (as citric acid)	——	0.7749
Ash	0.4859	0.1035

II. Chemical Changes of Mash during its Saccharifying and Fermenting.

In the spirit mash, as in the case of "Saké", the saccharification and the alcoholic fermentation takes place at the same time, but in the former case, these actions arise suddenly.

The analysis of mash is carried on 17 days during its ripening with the following results:

Date	Temp. of mash	PH	g in 100 c.c filtrate of mash		
			Acid (as citric acid)	Reducing sugar (as glucose)	Alcohol
1 (Jan. 11)	25°C	2.96	0.9645	2.2653	0.05
2 (〃 12)	24	2.87	1.6529	3.2020	3.46
3 (〃 13)	28	3.12	1.4866	0.4646	9.85
4 (〃 14)	27	3.24	1.4700	0.7630	11.72
5 (〃 15)	23	3.29	1.4434	0.8173	13.28
6 (〃 16)	17	3.36	1.4367	0.7841	14.00
7*(〃 17)	22	3.96	0.3559	1.7142	7.62
8 (〃 18)	30	4.07	0.2860	1.0295	8.46
9 (〃 19)	29	4.21	0.2661	0.4562	10.90
10 (〃 20)	25	4.26	0.2494	0.3269	11.49
11 (〃 21)	24	4.29	0.2594	0.2890	11.66
12 (〃 22)	22	4.26	0.2561	0.2798	11.52
13 (〃 23)	20	4.03	0.3193	0.3090	11.02
14 (〃 24)	18	4.09	0.2927	0.2731	11.08
15 (〃 25)	18	4.07	0.3093	0.2731	11.14
16 (〃 26)	16	4.12	0.2993	0.2731	11.14
17 (〃 27)	14	4.05	0.2993	0.2731	11.08

* Shows the second mixing of the raw materials.

III. Composition of Sweet potato spirit and its Byproduct.

General composition of the spirit prepared by above method, is as follows:

Specific gravity	0.9504	PH	6.01
Alcohol (vol. %)	40.38	Extract (g in 100 c.c.)	0.0317
Ash (g in 100 c.c.)	0.0182		

The distillated residue obtained from the mash, is used as fodder especially for pig, or manure; its analytical data are shown in the following table :

	g in 100 c.c. of original sample	In 100 parts of dry matter
Water	95.10	——
Dry matter	4.90	——
Crude protein	0.9402	19.19
Protein	0.7050	14.39
Crude fat	0.3123	6.37
Crude fibre	0.4664	9.52
Crude ash	0.4055	8.28
Extract	2.9000	59.18
Nitrogen free extract	2.7756	56.64

Summary.

(1) The titration acidity and hydrogen ion concentration of the "Awamori-koji" extract are very higher than those of the "Saké-koji" extract, that is :

	PH	g in 100 c.c.	
		as citric acid	as lactic acid
"Awamori-koji" extract	3.11	0.7749	——
"Saké" extract	5.49	——	0.0795

(2) The changes of the spirit mash during its ripening, are summarized as follows :

(a) The temperature of the mash is very high and the maximum reaches to 30°C or above.

(b) The titration acidity of the mash is very large, using the "Awamori-koji" as raw material. The organic acid in the mash is mainly citric, and moreover succinic acid.

(c) The hydrogen ion concentration of the mash is remarkably large, namely its PH value is below 3.0 at the beginning of the mash ; therefore the bacteria could not multiply in these medium even in a hot district. But by the second mixing of raw materials (sweet potato and water) the PH value of mash is elevated suddenly, on account of the phosphates in the sweet potato. In this time of the mash, the resinous substances in sweet potato played as an antiseptic. Thus the fermentation of mash may be carried out very safely.

(d) The amount of reducing sugar in the mash is very small, while on

the other hand the formation of alcohol is very active. From these facts, it may be said that, the sugar formed from the starch was changed to the alcohol immediately in succession.

(e) In the spirit mash a small amount of alcohol is lost nearly at the end of fermentation of mash by the evaporation. Then the distillation of mash should be performed at about the 5th day after the 2nd mixing of raw materials, when the content of alcohol reaches to the maximum.

Über Säuren und Alkohols im hochsiedenden Anteil des Fuselöls

Von

T. Taira

(Eingegangen am 3. März 1933.)

Bekanntlich ist das Fuselöl ein Nebenprodukt der alkoholischen Gärung, und wegen seiner Wichtigkeit für die Gärungschemie wie auch für die Hygiene ist es der Gegenstand zahlreicher Untersuchungen gewesen. Verhältnismässig gering ist aber die Anzahl der Arbeiten über die höher als Amylalkohol siedenden Anteile. Zwar wird angegeben, dass darin Fettsäuren wie Capron–, Oenanthyl–, Capryl–, Pelargon–, Laurin–, Myristin– und Palmitinsäure, ferner Alkohole wie n–Hexyl–, Isohexyl–, n–Heptyl–, Oktyl–, Nonyl– und Decylalkohol sowie Terpineol vorkommen, aber da bei bisherigen Arbeiten geringe Mengen Material verarbeitet und nur indirekte Methoden z. B. Elementaranalyse der betreffenden Fraktionen, Molekulargewichtsbestimmung und Analysen der Salze zum Nachweise benutzt wurden, so ist nach Ansicht des Verf. die Untersuchung durch direkte methoden sehr erwünscht.

Dem Verf. standen relativ grosse Mengen Material bei der Untersuchung des Fuselöls aus Rohrzucker–Melasse, Ipomoea und Kaoliang (in China einheimische Art Mohrenhirse der Familie Graminae) und des Reisbranntweins (rice brandy oil) zur Verfügung. Nachdem unter gewöhnlichem Druck die unterhalb 132°C siedenden Anteile abdestilliert worden waren, wurde der Rückstand verseift, um durch Extraktion desselben mit Äther die Seife vom unverseifbarem Anteil zu trennen. Nach Zersetzung der Seifenlösung mit Mineralsäure wurden die Fettsäuren mit Äther extrahiert. Die ätherische Lösung wurde dann mit geglühtem Natriumsulfat getrocknet und der Äther abdestilliert Die Fraktionierung des Rückstands wurde unter Benutzung des Widmer'schen Kolonnenapparats vorgenommen. Aus dem unter 10 mm bis 200°C übergehenden Anteile von einheitlichem Molekulargewicht wurden die Anilide hergestellt, um deren Schmelzpunkte zu bestimmen, ferner durch Mischprobe den Identitätsnachweis zu erbringen. Der unter 10 mm über 200° übergehende Anteil wurde nach dem Äther-Bleisalzverfahren behandelt, um die gesättigten von den ungesättigten Säuren zu trennen. Die gesättigten Säuren wurden dann einer fraktionierten Kristallisation aus Alkohol unterworfen, um schliesslich deren Schmelzpunkte und Molekulargewichte zu bestimmen. Von den ungesättigten Säuren wurden Bromadditionsprodukte hergestellt und so Hexa-, Tetra- und Dibromide erhalten und auf diesem Wege die einzelne ungesättigte Säure nachgewiesen. Die ätherische Lösung des unverseifbaren Produkts wurde mit geglühtem Natriumsulfat getrocknet, dann der Äther verjagt und der Rückstand unter Benutzung des Widmer'schen Kolonnenapparats fraktioniert. Jede Fraktion wurde noch einmal fraktioniert und so gereinigt. Dann wurden die physikalischen Konstanten jeder gereinigten Fraktion bestimmt. Sodann wurden die Fraktionen mit Chromsäuregemisch oxydiert und Semicarbazone aus den Oxydationprodukten gewonnen. Phenyläthylalkohol wurde durch Phenylurethan charakterisiert.

Tabell I.

(T. Taira, Journ. Agr. Chem. Japan IX, 7~18, 1933.)

	Fuselöl aus Rohrzuckermelasse	Ipomoea-Fuselöl	Kaoliang Fuselöl	Rice brandy oil
Capronsäure	4.7%	2.9%	+	(5)
Caprysäure	19.8	6.7	?	(2)
Caprinsäure	45.8	10.0	12.0%	(1)
Laurinsäure	5.3	7.2	?	(4)
Myristinsäure	0	0	?	(3)
Palmitinsäure	14.7	42.3	32.0	63.0%(roh)
Ölsäure	7.9	24.0	28.0	22.0
Leinölsäure	1.5	7.2		9.0
Linolensäure	0.3	0		0

Anmerkung: Die Klammern in der Rubrik "Rice brandy oil" der obigen Tabelle die Reihenfolge der Mengen. Beim Kaoliang-Fuselöl und Rice brand oil-Fuselöl ist der Gehalt der Myristinsäure sehr gering, sodass befriedigende Resultate nicht erhalten werden konnten.

Tabelle. II.

(T. Taira Joun. Agr. Chem. Japan, 1933, im Druck befindlich.)

	Methyl-n-amyl-carbinol	Methyl-n-heptyl-carbinol	Phenyläthyl-alkohol
Fuselöl aus Rohrzuckermelasse (36 L)	30 g	40 g	2 g
Ipomoea-Fuselöl (9 L)	2	3	16
Rice brandy oil (30 L)	ca. 2.0 g		6
Saké (27 L)	ca. 0.7 g		1

Von den bisher durch verschiedene Forscher als Bestandteile des Fuselöls angegebenen zahlreichen Fettsäuren können gesättigte Fettsäuren mit gerader Kohlenstoffzahl C_6 bis C_{16}, ferner die vom Verf. zuerst darin nachgewiesenen ungesättigten Fettsäuren mit gerader Kohlenstoffzahl, nämlich Ölsäure und Leinölsäure, als regelmässige Bestandteile des Fuselöls bestätigt werden. Aber Fuselöl aus Rohrzucker-Melasse und Ipomoea-Fuselöl enthalten keine Myristinsäure. Ferner enthält das Fuselöl aus Rohrzuckermelasse gering Mengen Linolensäure. Oenanthylsäure und Pelargonsäure, die bisher öfters als Bestandteile des Fuselöls angegeben wurden und die Fettsäuren mit ungerader Kohlenstoffzahl sind, konnten in keinem Fuselöl gefunden werden.

Die bisher im Fuselöl vermutenen primären normalen Alkohole und Alkohole der Isoform über C_6 sind auch nicht im unverseifbaren Teil enthalten. Sehr interessant ist die Tatsache, dass sekundäre Alkohole mit C_7 und C_9 darin vorkommen. Zwar fehlen in der Literatur Angaben, dass Phenyläthylalkohol, ein Gärungsprodukt des Phenylalanins, im Fuselöl vorkommt, doch ist dieser Alkohol im Ipomoea-Fuselöl, im Fuselöl des rice brandy oil und im Saké deutlich nachweisbar enthalten.

Dass sekundäre Alkohol des Fuselöls durch Phytoreduktion der durch β-Oxydation der Fettsäuren mit gerader Kohlenstoffzahl entstandenen Methylketone sich bilden, scheint wahrscheinlicher zu sein als die Ehrlich'sche Theorie einer Bildung durch Gärung der Aminosäure. Arbeiten über β-Oxydation durch Schimmel pilze sind zwar bekannt, aber letztere wurden nicht bei der Hefeentdeckt. Der Umstand, dass im Fuselöl aus Rohrzucker-Melasse, in welchem Fall Hyphomyceten nicht zur Wirkung kommen können, doch sekundäre Alkohole enthalten sind, scheint dafür zu sprechen, dass diese Wirkung der Hefe zukommt.

K. Shoji hat fast zur gleichen Zeit wie Verf. mitgeteilt, dass er aus rice brandy oil Methyl-n-amylcarbinol und Methylheptylcarbinol isolieren konnte.

(Scientific Papers of the Institute of Physical and Chemical Reserch. vol. 20 (1933) no. 405)

Nutritive Value of Sperm Whale Oil and Finback Whale Oil.

By Yoshikazu SAHASHI.

(Received February 10, 1933.)

Though nutritional studies of fats or oils have already been carried out by many investigators, little is yet attempted in the feeding experiments with such oils as sperm whale oil which contains a large amount of waxes.

The present author, therefore, carried some feeding experiments of rats with synthetic diets containing large amounts of the oils obtained from sperm whale (*Physeter macrocephalus* L.) and finback whale (*Balaenoptera physalus* L.) for the purpose of contributing something to this nutrition problem.

Through the previous work of M. Tsujimoto(1) and Y. Toyama(2), the chemical properties of the oils prepared from sperm whale and finback whale have already been determined as follows:

	The head oil from sperm whale (M. Tsujimoto)	The body oil from sperm whale (M. Tsujimoto)	Finback whale oil (Y. Toyama)
Sp. gr.	$d_4^{15} = 0.8848$	$d_4^{20} = 0.8806$	$d_4^{15} = 0.9231$
Refr. index	$n_D^{20} = 1.4633$	$n_D^{30} = 1.4620$	$n_D^{40} = 1.4727$
Acid value	0.99	1.24	2.21
Saponification value	147.1	131.6	196.6
Iodine value	71.4	82.4	112.9
Reichert-Meissel value	0.57	—	—
Unsaponifiable matter	36.0%	36.4%	1.09%
Fatty acids	65.0%	64.13%	—
Glycerol	3.52%	—	—
Cholesterol	0.18%		

The sperm whale oils consisted chiefly of mixed waxes. The unsaponifiable matter (wax alcohols) contains cetyl alcohol and oleic alcohol in nearly equal proportion. Small amounts of octadecyl and tetradecyl alcohol appear to be present. The total percentage of cholesterol was found to be 0.18% of the sperm oil. The fatty acids consisted of about 19% solid and about 81% of liquid acids. Among the solid (saturated) acids palmitic and miristic have been identified. A small quantity of caprylic or capric acids was also present. The liquid (unsaturated) acid consisted mainly of the oleic acids series: physetoleic and oleic acids. Not more than 1% of highly unsaturated acids was also present. On the contrary, the finback whale oils contained fatty acids of the same composition which occur in other animal or vegetable oils as common glycerides.

The present author carried out feeding experiments with a genuine head oil and an intestine oil obtained from sperm whale (male: body length about 14 meters) and with a blubber oil and an intestine oil prepared from a finback whale. These samples were kindly supplied from Tōyō Hogei Kaisha.

Experimental.

(1) *Experiments with diets containing whale oils in sufficient quantity.*

The analysis of the samples used in the experiments:

	Sperm whale		Finback whale	
	The head oil	The intestine oil	The blubber oil	The intestine oil
Sp. gr.	——	——	$d_4^{15} = 0.916$	$d_4^{15} = 0.916$
Refr. index	——	$n_D^{23} = 1.464$	$n_D^{22} = 1.466$	$n_D^{22} = 1.467$
Acid value	0.9	2.0	0.2	0.9
Sap. value	145.4	144.4	185.5	180.5
Iod. value	62.7	88.6	92.7	97.6
Vitamin A	——	——	——	——

For the experiments: Albino rats weighing 40∼50 g each, were previously fed for several days on a complete diet until they reached 50∼60 g. Then they were divided into several groups, each consisting of four animals, and were fed on various experimental synthetic diets. (Fig. 1∼8)

The diets consisted of:

Potato starch (Japanese Pharmacopoea IV)	65 g	60 g
Fish meat protein (bonito meat freed from fat)	15 ″	15 ″
McCollum's salt mixture	4 ″	4 ″
Oryzanin solution (Sankyo & Co.)	5 cc	——
Dried yeast (supplied from Oriental Company extracted with ether)	——	2 ″
Whale oils	**15 g**	**20 g**
Biosterin, dissolved in olive oil, given per os daily.	1 mg	1 mg

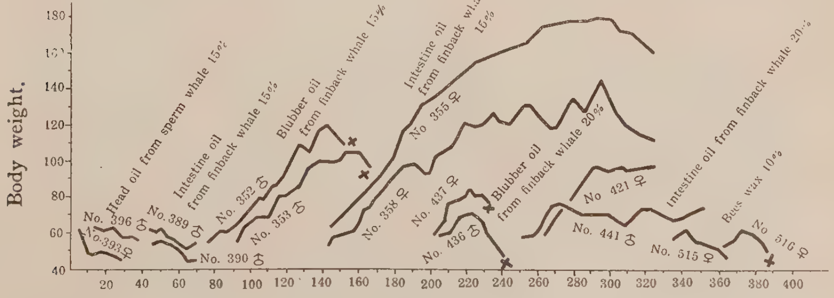


Fig. 1. The growth curves of the rats fed on diets containing whale oils.

(II) *Experiments with waxes and purified sperm oils.*

In order to investigate the characteristic symptom of rats fed on the diets containing sperm oils as described above, the author attempted to purify the waxes from sperm oils.

i) Spermaceti (cetin): When the head oil from sperm whale was cooled at 0~5°C, a large quantity of the solid wax which consisted chiefly of cetin separated at the bottom as white crystalline mass. It was collected, freed from the mother liquor (so-called winter sperm oil) by centrifugal machine, and recrystallized from hot alcohol.

ii) Liquid waxes: 100 g said winter sperm oils freed from the above solid waxes were boiled with 400 c.c. of a 3% Na_2CO_3 solution for 3 hours. The impurities in the oils are thus completely dissolved. After cooling, the liquid oils were separated and washed with water until the wash water contained no trace of sodium carbonate. Analysis of waxes:

	Solid waxes	Liquid waxes		
	Spermaceti (cetin) (head oil)	Winter sperm oil, freed from spermaceti (head oil)	Winter sperm oil, treated with Na_2CO_3 (head oil)	Winter sperm oil, treated with Na_2CO_3 (intestine oil)
Sp. gr.	——	$d_4^{15} = 0.887$	$d_4^{15} = 0.856$	$d_4^{15} = 0.917$
Refr. index	——	$n_D^{26} = 1.459$	$n_D^{26} = 1.460$	$n_D^{28} = 1.470$
Acid value	0	0.5	0.2	0.2
Sap. value	135.3	152.8	153.4	148.3
Iod. value	17.0	76.9	66.2	90.6

iii) Finback whale oils purified with Na_2CO_3: The analysis of the samples treated with Na_2CO_3 in the above method (ii):

	Purified finback whale oils	
	The blubber oil	The intestine oil
Sp. gr.	$d_4^{15} = 0.919$	$d_4^{15} = 0.918$
Refr. index	$n_D^{28} = 1.474$	$n_D^{28} = 1.474$
Acid value	0.2	0.2
Sap. value	184.3	182.5
Iod. value	93.0	102.5

The results indicated that the glycerides are not destroyed.

Feeding experiments: Feeding experiments were carried out in the similar manner as described above. The diets consisted of:

Potato starch (J. P. IV.)	70 g	65 g
Fish meat protein (bonito meat)	15″	15″
McCollum's salt mixture	4″	4″
Yeast as described above	2″	——
Oryzanin solution	——	5 cc
Waxes prepared as described above	**10 g**	**15 g**
Biosterin dissolved in olive oil per os daily	1 mg	1 mg

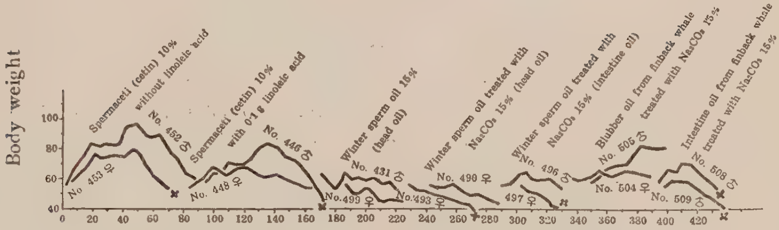


Fig. 9. The growth curves of the rats fed on diets containing purified whale oils.

Besides, a little linoleic acid was added to each diet, because it was proved to be an indispensable food factor, the absence of which causes a disease like avitaminosis.

From the above experiments it can be seen that the pure waxes have no noticeable nutritive value upon rats, and seborrhoea seems sometimes to be produced by other fats or oils which contain waxes either in small amount or free from it (Fig. 9∼14).

(III) *Experiments with fatty alcohols.*

Pure waxes had neither any pronounced nutritive value upon rats, nor seemed to produce seborrhoea. So, the authors carried out some experiments with purified fatty alcohols prepared from the head oil (sperm whale) by saponifiction. As it was shown by M. Tsujimoto and Y. Toyama, the principal components of the head oil from sperm whale are cetyl, octadecyl and oleic alcohols, so the authors prepared these alcohols in pure state and separately tested on rats.

Cetyl alcohol: The solid waxes isolated from sperm oil was saponified with alc. KOH in the usual way, and the fatty acids were precipitated as Ca-soaps with hot alcoholic solution of $CaCl_2$; filtered and the filtrate, after the removal of ethyl alcohol, was extracted with ether, and the etherial extract after washing with water was evaporated to dryness. The residue consisted chiefly of cetyl and octadecyl alcohols. It was recrystallized from acetone, and converted into the corresponding acetate by boiling with three times its amount of acetic anhydride for three hours. The acetylated products thus obtained were fractionally distilled in vacuum. The fraction boiling at

166∼7°C (2 mm) having the iodine value 7.4∼10 was saponified and subjected to distillation and the distillate was used for animal experiments; b p (3 mm)=161∼2°C. In order to determine the purity of this preparation, it was again converted into acetate and the iodine value was determined with the following results.

0.1215 g Subs. gave 1.1 c.c. $Na_2S_2O_3$ (1 c.c.=12.58 mg iodine) iodine value=11.4
0.1206 〃 〃 〃 1.1 〃 〃 〃 〃 =11.4

Oleic alcohol: Oleic alcohol was prepared in the similar manner as that of cetyl alcohol from the aceton-soluble portion of the nonsaponifiable matter, separated from the liquid waxes in sperm oils. After repeated distillation of the acetylated product, the preparation gave the following constants: b p (4 mm)=180∼5°C, $d_4^{15}=0.894$, $n_D^{20}=1.4532$, iodine value=69.5∼79.5 (cal. 81.9). The preparation used in the animal experiment was also prepared from the above one by saponifying and redistilling: b p (4 mm)=178∼180°C. The purity of the preparation was confirmed by converting it into the acetate: b p (3 mm)=180∼182°C.

0.1148 g Subs. gave 6.5 c.c. $Na_2S_2O_3$ (1 c.c.=12.58 mg iodine) iodine value=71.2
0.1185 〃 〃 〃 6.7 〃 〃 〃 〃 =71.1

Oleic alcohol (synthetic): Synthetic oleic alcohol was prepared from ethyl oleate by reduction with Na in dry ethyl alcohol. After the removal of alcohol the sample thus obtained was washed with alkali, dried over anhydrous Na_2SO_4, and distilled in vacuo: b p (4 mm)=177∼8°C. Then, the preparation was analyzed as the acetate: b p (3 mm)=180∼2°C, $d_4^{15}=0.889$, $n_D^{20}=1.4543$.

0.1139 g Subs. gave 7.8 c.c. $Na_2S_2O_3$ (1 c.c.=12.58 mg iodine) iodine value=86.1
0.1133 〃 〃 〃 7.8 〃 〃 〃 〃 =86.6
(cal. 81.9)

Feeding experiments: The feeding experiment of rats with the fat-deficient diet supplemented with 5% and 10% fatty alcohols were as follows: The diets consisted of:—

Potato starch (J. P. IV.)	70 g	75 g	80 g
Bonito meat protein	15 g	15 g	15 g
McCollum's salt mixture	4〃	4〃	4〃
Yeast as above described	2〃	2〃	2〃
Fatty alcohol (cetyl or oleic alcohol)	**10 g**	**5 g**	**0 g**
Biosterin dissolved in olive oil per os daily	1 mg	1 mg	1 mg

Preparation used in the expt.	Body wt. at the beginn. of the expt. (av. of 4 rats)	Body wt. at the end of the expt. (av. of 4 rats)	Days of living (av. of 4 rats)
Cetyl alcohol 10%	59 g	44.0 g	11.0
(the head oil f. sperm whale) 5%	58.5 g	45.5 g	10.0

Oleic alcohol	10%	58 g	44.0 g	3.5
(〃)	5%	59 g	45.7 g	3.7
Oleic alcohol (synthetic)	10%	58 g	40.0 g	5.0
Control	0%	61 g	105.0 g	50.0

Moreover, in order to determine the noxious effect of oleic alcohol on rats, when fed on a diet containing butter, rats were fed on a basal diet consisting of starch 75 g, bonito meat protein 15 g, butter 5 g, McCollum's salt mixture 4 g, and commercial oryzanin solution 5 c.c.. The sample oleic alcohol 0.1 g was supplied for each rat per os daily.

The preparation used in the expt.	Initial body wt.	Max. body wt. during the exp.	Body wt. at the end	Duration for expt.
Oleic alcohol	61 g	66 g	63 g	20 (days of living)
(the head oil sperm whale)	57 g	63 g	53 g	21 (〃)
Control	54 g	69 g	69 g	25

From the above result, it was confirmed that free fatty alcohols especially oleic alcohol gave toxic effects upon animals.

(IV) *Injection experiments with oleic alcohol.*

This experiment was conducted with the object of determining the possible role of oleic alcohol in the production of seborrhoea in rats fed on the diet supplied with sperm oils. 2 c.c. oleic alcohol or its acetate prepared from the sperm oil was injected to the peritoneal cavity of rats which were previously fed on an adequate diet. No seborrhoea was produced in any of the rats (4 rats); and three of them died after 10～40 hours and the last one injected with 2 c.c. oleic acetate died after 6 days. So it was also concluded that oleic alcohol itself would not produce seborrhoea in rats.

(V) *Effect of linoleic acid upon seborrhoea of rats supplied with sperm whale oils.*

An additional experiment was repeated to see the effects of linoleic acid and yeast on the sick rats, because they were recently recognized to be an indispensable food factor like vitamins. Rats were fed on the similar diet as above described: potato starch 65 g, fish meat protein 15 g, McCollum's salt mixture 4 g, dry yeast extracted with ether 2 g, sperm whale oil or finback whale oil 15 g, and in addition to the basal diet biosterin 1 mg and pure linoleic acid* 50∼100 mg were supplied to every rat per os daily. The rats given the head oil from sperm whale exhibited seborrhoea after 3 weeks; but on the contrary, the one given the intestine oil from sperm whale and finback whale oils was not observed to show the skin symptom for the entire feeding period of about 100 days. (Fig. 15∼16).

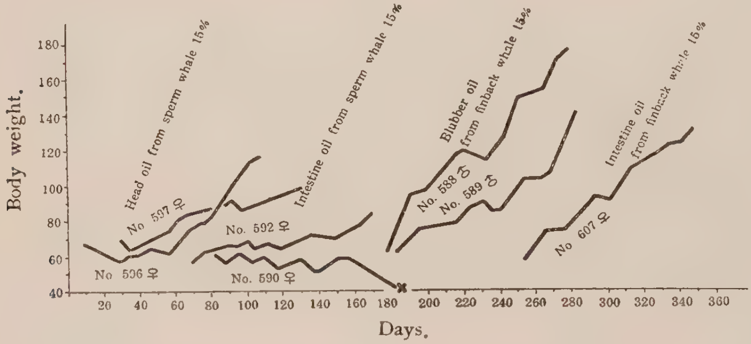


Fig. 15 The growth curves of rats fed on diets containing 15% sperm whale or finback whale oils, supplemented with a little linoleic acid and dry yeast.

Summary

1. The sperm whale oils produced seborrhoea in rats and exhibited the retarding action upon the growth of rats. Finback whale oils (especially the intestine oil), on the contrary, indicated a pronounced nutritive value.

2. In order to determine whether waxes might exhibit any nutritive activity in rats, the head oil from sperm whale was freed from spermaceti (cetin) at a temperature of 0∼5°C, and expressed oil was used as the sample of winter sperm oil for investigation. Moreover, the above four kinds of oils were separately treated with Na_2CO_3 in boiling water, and the refined oils so obtained were used for the experiments. Pure waxes prepared from sperm oil when given per os did not show the said symptom, especially when a little linoleic acid and dry yeast were administered with the waxes; so that, seborrhoea developed in rats fed on the diets containing large amount of sperm oils does not seem to be due to the high content of waxy substances in the materials.

3. Fatty alcohols especially unsaturated fatty alcohols like oleic alcohol which is a component of sperm whale waxes, have marked toxic property, but do not produce seborrhoea in rats when given per os or injected. As the waxes as such had no noxious effect, it was concluded that the waxes were not assimilated by the animals; otherwise, the diet containing sperm

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* Pure linoleic acid was specially prepared by the present author from soy bean oil by the bromination method.

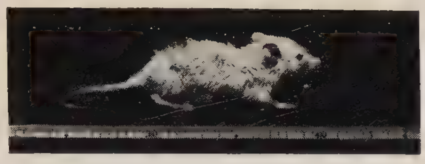

Fig. 2—Head oil from sperm whale 15% No. 363. body wt. 49 g (after 9 days)

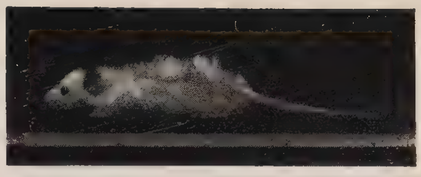

Fig. 3—Intestine oil from sperm whale 15% No. 390. body wt. 55 g (after 11 days).

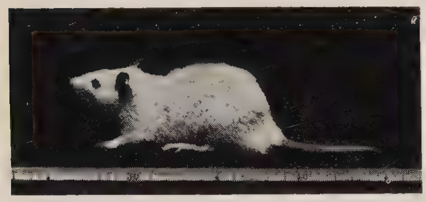

Fig. 4—Blubber oil from finback whale 15% No. 353. body wt. 102 g (after 48 days).

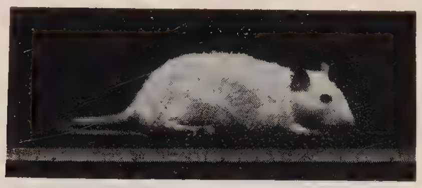

Fig. 5—Intestine oil from finback whale 15% No. 355 body wt. 121 g (after 48 days).

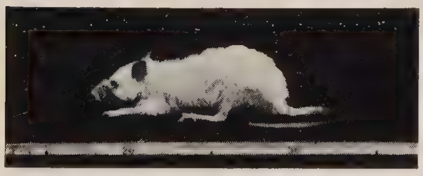

Fig. 6—Blubber oil from finback whale 15% No. 351. body wt. 64 g (after 75 days).

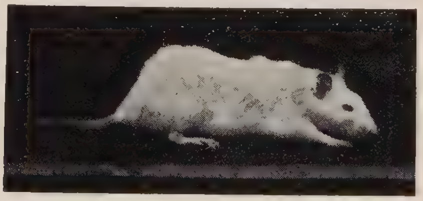

Fig. 7—Intestine oil from finback whale 15% No. 355. body wt. 152 g (after 75 days).

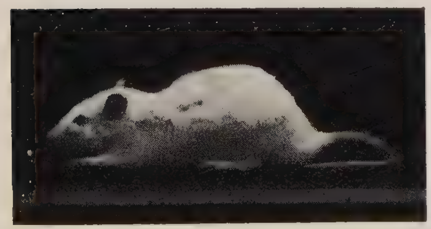

Fig. 10—Spermaceti (cetin) 10% added with yeast 2% No. 452. body wt. 89 g (after 52 days).

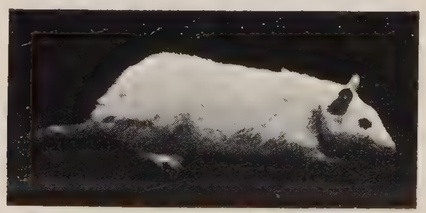

Fig. 8—Intestine oil from finback whale 15% No. 355. body wt. 180 g (after 128 days).

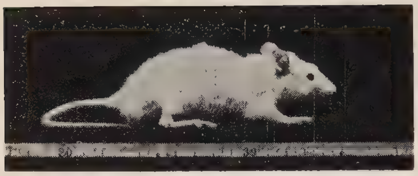

Fig. 11—Spermaceti (cetin) 15% Oryzanin 5 cc added No. 428 body wt. 65 g (after 30 days).

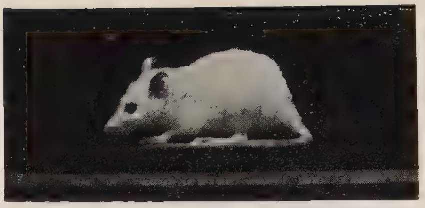

Fig. 12—Head oil from sperm whale 15% Oryzanin 5 cc added No. 490 body wt. 51 g (atter 30 days).

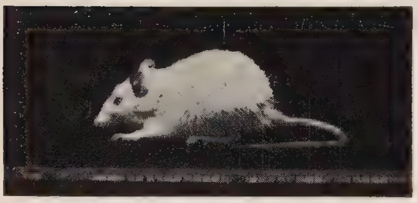

Fig. 13—Intestine oil from sperm whale 15% treated with Na_2CO_3 Oryzanin 5 cc added No. 496 body wt. 61 g (after 30 days).

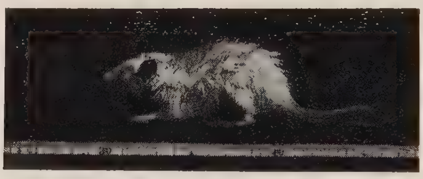

Fig. 14—Blubber oil from finback whale 15% treated with Na_2CO_3 Oryzanin 5 cc added No. 504 body wt. 62 g (after 50 days).

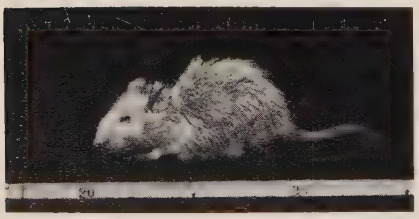

Fig. 16—Head oil from sperm whale 15% added with linoleic acid (2 drops) and yeast No. 595 body wt. 53 g (after 3 weeks).

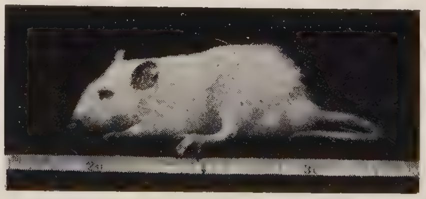

Fig. 17—Intestine oil from sperm whale 15% added with linoleic acid (2 drops) and yeast No. 592 body wt. 69 g (after 5 weeks).

whale oils in sufficient quantity should give more noxious effect.

Moreover, the author has observed that diets supplied with fat consisting of ordinary glycerides have frequently produced the similar symptom in the skin of rats; and on the other hand, that the symptom was prevented by the addition of a small amount of yeast and linoleic acid even when given sperm oils.

The author expresses his sincere thanks to Prof. U. Suzuki for his kind advice and encouragement throughout the work. The author is also indebted to Mr. K. Tago and to the Tōyō Hogei Kaisha for the kind supply of reliable whale oils in fresh condition.

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(1) J. Soc. Chem. Ind. Japan, Suppl. **24**, 11~15, (1921).
(2) J. Soc. Chem. Ind. Japan, Suppl. **28**, 13~16, (1925); **30**, 137~8, (1927).

On the Ergosterin Content of Various Edible Mushrooms in Japan.

By Midzuho SUMI.

(Received February 17, 1933.)

Some years ago(1), the author had isolated ergosterin from *Cortinellus Shiitake*, carefully studied its chemical nature, converted it into vitamin D by irradiation with ultra-violet rays, and proved it to be identical with the preparations obtained from ergot or from yeast by other authors. Later, it was observed that this substance is widely distributed in many other mushrooms, either cultivated or grown wild in various districts of Japan. As these mushrooms are not only consumed to a large extent as a favourite food by our people but also some of them are exported to other countries in a notable amount, it seems desirable, from the stand point of nutrition and public health, to determine their ergosterin content quantitatively. As there is not yet reliable method known for this purpose the author has adopted the digitonin method which is commonly used for the determination of sterins.

Since there is no noticeable amount of sterin other than ergosterin, detected in these fungi, the author presumes that the result obtained by the above method will suffice to represent the approximate amount of ergosterin.

The method of estimation is as follows:

1) The dried and finely powdered material (50 g for each determination) is thoroughly extracted with ether in Soxhlet apparatus, the etherial extract is then evaporated and the residue thus obtained is dissolved in 95% alcohol and filled up to a definite volume.

2) A portion of this solution serves for the determination of free ergosterin. For this purpose, it is treated with an excess of 1% digitonin solution in 90% alcohol, the free ergosterin being thus completely thrown down as the digitonid. After standing overnight in a cool place, the precipitate is collected on a weighed filterpaper, washed with alcohol and ether and dried at 100° to constant weight. From the weight of the digitonid, the percentage of free ergosterin in the original sample is calculated.

3) For the determination of total ergosterin, a part of the latter which exists in the ester form must be previously saponified; otherwise, it is not precipitated by digitonin. For this purpose the above alcoholic solution is boiled with 20% caustic potash for one hour, and after cooling, it is diluted with water and thoroughly extracted with ether. The ergosterin together with other unsaponifiable matters is thus taken up in ether. The etherial extract thus obtained is, after dehydration, evaporated and the residue is again dissolved in 95% alcohol and treated with the digitonin solution as stated above. The difference between the total and the free ergosterin represents the quantity of ergosterin in ester form. For yeasts and spores of *Aspergillus oryzae*, the above method cannot be directly applied, so it is modified as follows;

The dried material (50 g) is suspended in 200 c.c. of 95% alcohol and boiled for 2 hours with concentrated caustic potash (40 g). The supernatant solution is decanted and then the insoluble residue is again boiled with caustic potash likewise. The combined extract thus obtained is then evaporated at low temperature, diluted with a large quantity of water and thoroughly extracted with ether. The etherial solution is dehydrated, evaporated and the residue is dissolved in 95% alcohol, filled up to a definite volume, and treated with the digitonin solution as stated above. In this way, the amount of total ergosterin is obtained.

I. Ergosterin Content in different Parts of Cortinellus Shiitake.

Full grown "Shiitake" was divided into three parts, i. e.,

a) Flesh together with peel. b) Gills. c) Stem.

Each part was separately dried, powdered and analyzed with the following results.

Table. I.

Part of body	Relative wt. of each part of fungus	Ergosterin % in		
		Free state	Ester form	Total
a	37%	0.2261	0.9233	0.2494
b	43	0.3013	0.0312	0.3325
c	20	0.1324	0.0136	0.1460

We see from the above result that the ergosterin content is highest in gill while lowest in stem.

II. Ergosterin Content of *Cortinellus Shiitake* at Different Stages of Growth.

The samples for analysis were gathered at four different stages, i. e.,

a) Button stage :– Cap was globular cylindrical form, the diameter of which was 5∼6 mm.

b) Young stage :– Cap expands and veil separates from the margin, the diameter 1∼2 cm.

c) Mature stage :– Cap is broadly expanded or nearly flat, the diameter of which was *ca.* 4 cm. In this stage the fungus is usually harvested.

d) Old stage :– Over–riped. Cap becoming funnel shaped; the diameter was more than 5 cm.

The analysis of these samples gave the following results.

Table II.

Stage of growth	Ergosterin % in		
	Free state	Ester form	Total
a	0.1572	0.0214	0.1786
b	0.1882	0.0238	0.2120
c	0.2353	0.0286	0.2639
d	0.2525	0.0325	0.2850

The above result shows that the ergosterin content increases with the development of the fungus.

III. Ergosterin Content of Various Mushrooms.

Most of the samples used in this experiment was kindly supplied by Dr. S. Mimura, to whom the author expresses his hearty thanks. For analysis the dried and powdered material was treated as stated above. The results were as follows :–

Table III.

Name	Fresh sample %		Dry Matter Ergosterin %
	Moisture	Ergosterin	
Armillaria Matsudake Ito et Imai	35.32	0.0309	0.2103
Lactarius Hatsudake Tanaka	91.56	0.0162	0.1915
Tricholoma Shimeji Kawamura	93.42	0.0147	0.2237
Armillaria mellea (vahl) Quelet	87.31	0.0158	0.1243
Boletus edulis (Bull) Fr.	18.72	0.1017	0.1252
Psalliota campestris (Linn) Fr.	91.85	0.0142	0.1751
Rhizopogon rubescens Tul.	73.20	0.0424	0.1582
Tricholoma colossum Fr.	88.23	0.0204	0.1734
Cortinellus Shiitake P. Henn (*Cortinellus Berkeleyanus* Ito et Imai)	70.87	0.0769	0.2639
Polyporus frondosus (Fl. Dan.) Fr.	89.50	0.0106	0.1008
Pholiota discolor peck. Young	91.50	0.0164	0.1932
Pholiota discolor peck. Mature	90.30	0.0228	0.2352
Polyporus varius (pers) Fr.	16.55	0.0778	0.0932
Pleurotus ostreatus (Jacq) Sacc.	10.53	0.1277	0.1428
Hydnum aspratum Berk.	13.10	0 2331	0.2683
* *Polystictus versicolor var nigricans* Losch.	17.46	0.0423	0.0513
(2) Spores of *Aspergillus oryzae*	17.82	0.3304	0.4021
** Dried Baker's yeast	7.87	0.3369	0.3657

Conclusion.

The ergosterin content of *Cortinellus Shiitake* varies for different parts of body and it increases a little as the latter grows.

By comparing the ergosterin content of various kinds of edible mushrooms, it was found that they range between 0.1 to 0.4% of the dry materials.

The author wishes to express his sincere thanks to Professor U. Suzuki for his kind guidance throughout this work, and also to Dr. S. Mimura for many valuable advices and conveniences.

Reference

(1) Biochem. Z., **204**, 397, (1929); Proc. Imp. Acad., **4**, No. 3, (1928).
(2) Biochem. Z., **195**, 161, (1928).

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* *Polystictus versicolor var nigricans* does not belong to the edible mushroom; it grew on the decayed oak stump after Shiitake was harvested.

** As the spores of *Aspergillus oryzae* and yeast contain besides ergosterin many other sterins, the figures given here represent only approximate values.

H. Wieland and M. Asano: Ann., **473**, 300, (1929).
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H. Wieland and W. Stanley: Ann., **489**, 31, (1931).
R. Takata: J. Soc. Chem. Ind. Japan., **32**, 908, (1929).

Studies on Experimental Rickets. IV

On the Occurrence of Ergosterol in Shoyu Oil.

By

Seiichi IZUME, Yoshinori YOSHIMURA and Yasushi HIDAKA.

(From the Central Laboratory, South Manchuria Railway Co., Dairen, Manchuria)

(Received April 4, 1933.)

Introduction.

Shoyu oil is an oily substance which rises to the top of shoyu or soy sauce when "moromi"* is filtered by means of a hydraulic press. The oil is therefore obtainable as a by-product of soy sauce brewing, and its annual production may be estimated at about 100,000 koku or 16,000 tons in Japan proper alone. It is a dark brown oil with a pungent soy sauce-like smell, and its boiling point is considerably lower than those vegetable oils, which is due to the fact that a majority of its fatty acids is combined with ethyl alcohol instead of glycerine. The use of shoyu oil is rather limited owing to its unpleasant odour and low boiling point, the oil being at present utilized almost exclusively as a raw material for the manufacture of powdered soap of cheaper grades.

During the course of spectrographical and biological examinations of sterols isolated from different sources, we found that a significant amount of ergosterol was present in the sterol obtained from shoyu oil. We thought therefore that it might be of some economical and scientific significances to determine the value of shoyu oil as a raw material of obtaining vitamin D preparations. Accordingly we tried to estimate the ergosterol content of shoyu oil supplied by several different brewers and then to demonstrate on the animal its profound antirachitic activities conferred by ultra-violet irradiation. We also discussed in the present paper the origin of ergosterol found in shoyu oil, and speculated on its economical value as a source of ergosterol in comparison with various other substances containing ergosterol.

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* For the brewing of soy sauce tane-koji or the culture of aspergillus oryzae is added to a mixture of nearly equal parts of cooked soya bean and roasted wheat, and allowed to stand in a cellar until mixture is slightly covered with the fungus, which is then steeped in a large vat of brine and left to ferment for about a year. After the ripening period the fermented mass which is called "moromi" is transferred into a press and liquid sauce is pressed out.

Experimental Results.

(A) Chemical Examinations.

The specimens of shoyu oil supplied by four different brewers had the general characteristics given in Table I.

Table I.

	I	II	III	IV
Specific gravity (15°C)	0.892	0.899	0.896	0.896
Acid value	64.2	61.6	57.6	48.5
Saponification value	183.1	183.3	184.4	183.2
Reichert-Meissl value	——	0.33	——	——
Iodine value (Wij's)	112.5	117.3	117.600	125.8
Unsaponifiable matter	3.03%	3.87%	2.72%	4.130%

Sterol was isolated from the unsaponifiable matter by means of recrystallizations by using acetone and then alcohol as solvents (yield, about 2% of the oil). It gave an intense colour with the Liebermann–Burchard's reagent and also it possessed the Rosenheim's chloral hydrate and trichloroacetic acid reactions both specific for ergosterol. A specimen of sterol melted at 136∼7°C and showed $[\alpha]_{5461}^{25^\circ}=-12.85^\circ$, while its acetate had m.p. of 131∼2°C and $[\alpha]_{5461}^{24^\circ}=-13.35^\circ$.

(B) Spectrographical Examinations.

The spectrographical examinations were carried out on the ether solutions of both shoyu oil and its sterol, employing a hydrogen vacuum tube as the source of ultra-violet light. It was observed that both of these materials had maximum absorption bands at 293, 282, 270 and 260 $\mu\mu$; the positions being identical with those of ergosterol.

The estimation of ergosterol content of test materials was based on the concentration as well as on the thickness of absorption layer of their solutions at which the 282 $\mu\mu$ band shows the similar intensity to that given by a standard solution of ergosterol at a certain thickness. Four specimens of shoyu oil above mentioned were observed to contain ergosterol at 1.1, 1.3, 1.4 and 1.1%: and three specimens of sterol separated from different samples contained ergosterol at 19.8, 16.7 and 20.0%.

(C) Biological Examinations.

The antirachitic properties of test materials were demonstrated on rats, following the method previously reported(1). It was observed, as indicated in Table II, that while the non-irradiated shoyu oil had no appreciable degree of antirachitic properties, the same oil after one hour's exposure to the light of a mercury vapour lamp became exceedingly great in the above properties, the daily doses of 1/10 mg being sufficient to produce a constant healing of experimental rickets in rats. The sterol similarly irradiated with ultra-violet light was found to be more potent in the activities, doses such as 1/100 mg per day being always effective in curing rickets.

Table II.

Test material	Daily dose	Rat No.	Radiographic findings at the end of	
			Rachitic period	Test period
Shoyu oil	10% of diet	BBN02	Moderate rickets	No healing
	〃	BBN10	〃	〃
	5 % 〃	BBN12	〃	〃
	〃	BBN21	〃	〃
	100 mg	BBL02	〃	〃
	〃	BBN10	〃	〃
Irradiated shoyu oil	10 mg	BBC00	Slight rickets	Complete healing
	〃	BBC01	Moderate rickets	〃
	〃	BBC12	〃	〃
	〃	BBC21	Slight rickets	〃
	1 mg	BBK00	Moderate rickets	〃
	〃	BBK01	〃	〃
	〃	BBK02	Slight rickets	〃
	〃	BBK10	Moderate rickets	〃
	0.5 mg	BBL00	〃	〃
	〃	BBL01	Marked rickets	〃
	〃	BBN00	Slight rickets	〃
	〃	BBN01	〃	〃
	0.1 mg	BBR00	Moderate rickets	〃
	〃	BBR01	〃	Advanced healing
	〃	BBR02	〃	〃
	〃	BBR10	〃	Complete healing
Irradiated sterol	1 mg	AMF02	Moderate rickets	Complete healing
	〃	AMF10	Marked rickets	Advanced healing
	〃	AMG00	Moderate rickets	Complete healing
	〃	AMG01	Marked rickets	〃
	0.1 mg	AME02	Moderate rickets	〃
	〃	AME10	Marked rickets	〃
	〃	AMF00	〃	〃
	〃	AMF01	Slight rickets	〃
	0.01 mg	AMD02	Moderate rickets	〃
	〃	AMD10	Marked rickets	Slight healing
	〃	AME00	Moderate rickets	Complete healing
	〃	AME01	〃	〃
	0.001 mg	AMQ00	〃	Advanced healing
	〃	AMQ01	〃	Slight healing
	〃	AMQ02	〃	Advanced healing
	〃	AMQ10	Slight rickets	Complete healing

Discussion

The sterol of shoyu oil was first examined by Tsujimoto and Ueno(2) and then by Fukai(3). These investigators were all of the opinion that it was nothing but phytosterol. Fukai(4) lately investigated more thoroughly on the sterol obtained from the oil of shoyu press-cake and was able to isolate two kinds of sterols. The one metled at 95∼97°C and showed $[\alpha]_D^{19°}=+20$, and the other had m.p. of 137.5∼140°C and $[\alpha]_D^{19°}=-44$. Based on these facts as well as on the results of elementary analyses of the sterols he conjectured that coprosterol might be present in the former substance and ergosterol in the latter.

The results of biological investigations conducted by us were found to be consistent with our spectrographical findings, which showed that in shoyu oil more than 1% of ergosterol was present. It is rather surprising to find that shoyu oil contains ergosterol in such large quantities, which interested us to speculate on its origin. As above cited, the chief organic raw materials used for the brewing of soy sauce are soya bean and wheat in which oils are contained at about 18 and 2% respectively. We previously proved that ergosterol was present in soya bean oil⁽⁵⁾ at about 0.0005∼0.001% and in wheat bran oil at about 0.05∼0.1%. A mixture of equal parts of soya bean and wheat is therefore capable of supplying oil which contains ergosterol at a level of about 0.0055∼0.011%, a great discrepancy in the ergosterol content being thus found between the oil supplied by the mixture and shoyu oil. It is therefore reasonable to suppose that a greater part of ergosterol found in shoyu oil is derived from the sources other than these raw materials.

The micro-organisms which participate in the soy sauce brewing, such as, aspergillus oryzae, various yeasts (chiefly zygosaccharomyces) and bacteria are shown by a number of investigators to contain ergosterol in their bodies. It is also shown by Takahashi and Lin⁽⁶⁾ that aspergillus oryzae can synthesize ergosterol from the nutrient media containing no organic substances except sucrose. It is concluded from these facts and considerations that a major part of ergosterol in shoyu oil may be produced by various micro-organisms during the period of fermentation, only a minor part being derived from the raw materials.

The following substances may be regarded as important raw materials which can be utilized for the commercial production of vitamin D preparations in Japan: beer yeast, ergot, tane-koji or the culture of aspergillus oryzae, cotinellus shiitake, armillaria edodes, saké press-cake, wheat bran and rice polishings. The Dairen market price and ergosterol content of these materials as well as the cost of materials equivalent to 1 kg of ergosterol were compared with those of shoyu oil. These comparisons are shown in Table III, from which it must be admitted that shoyu oil is capable of supplying ergosterol much cheaper than any of the other substances above mentioned. We therefore think that shoyu oil will be highly esteemed in the near future as one of the most valuable sources of obtaining vitamin D in the orient.

Table III.

Raw Material	Price of material per kg (yen)	Ergosterol content (%)	Cost of material equivalent to 1 kg of ergosterol (yen)	Investigator
Shoyu oil	0.16	1.0 ∼1.5	10.7∼16.0	Authors
Beer yeast (dry)	2.67	0.2 ∼0.4	668∼1,335	Sumi, Authors
Ergot	4.00	0.13	3,077	Hart and Heyl
asp. oryz. (dry)	——	0.25	——	Takahashi and Lin
Spores of asp. oryz.	——	0.08	——	Sumi
Tane-koji (dry)	2,50	0.05 ∼0.08	3,125∼5,000	Authors
Cortinellus shiitake (dry)	3.00	0.26	1,153	Sumi
Armillaria edodes (dry)	2.70	0.21	1,286	Sumi
Saké press-cake (dry)	0.45	0.01 ∼0.02?	2,250∼4,500	Takahashi and Lin
Wheat bran	0.03	0.003∼0.006	500∼1,000	Authors
Rice polishings	0.04	0.002∼0.004	1,000∼2,000	Authors

Summary.

(1) It was spectrographically estimated that several specimens of shoyu oil supplied by different soy sauce brewers contained ergosterol at a level of about 1.1∼1.4% and those of sterol isolated from the oil at about 16.7∼20.0%.

(2) Both shoyu oil and its sterol were shown to be rendered highly antirachitic by means of ultra-violet irradiation, daily doses of 1/10 mg of irradiated oil and 1/100 mg of irradiated sterol being sufficient to produce a constant healing of rickets in rats.

(3) There are several evidences to indicate that a major part of ergosterol found in shoyu oil can be produced by aspergillus oryzae and other micro-organisms during the fermentation and only its minor part to be derived from the raw materials of soy sauce.

(4) It is suggested that shoyu oil can be utilized as the cheapest source of ergosterol in Japan among the known substances containing ergosterol.

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Studies on Blood Pressure Decreasing Substances.

Part I. The presence of blood pressure decreasing substances in yeast cell.

By

Masao Toki, Tohkichi Miyoshi and Nagao Uyeda.

(Received April 17, 1933.)

When dried yeast (Sac. cereviciae) is administrated orally per kg ca. 0.05 g to a rabbit the blood pressure is more or less decreased.

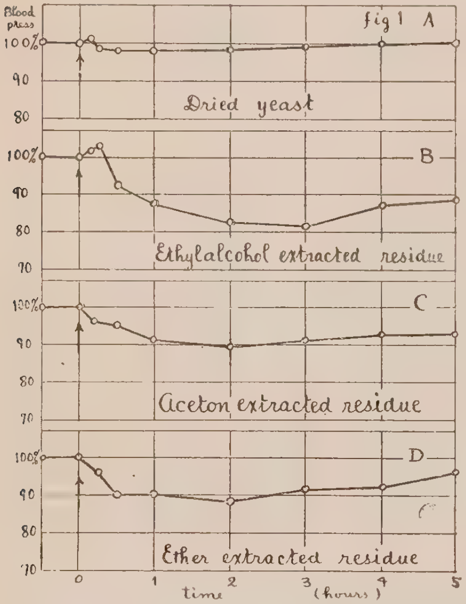

Fig. 1 Effects of dried yeast and its organic solvent insoluble part on the blood pressure of rabbit, per kg 0.05 g per os.

This blood pressure decreasing activity is pronounced in some degree by extracting with organic solvents such as alcohol, acetone, ether etc. as shown in fig. I.

It is proved also true in the case of ten different species of yeast as shown in fig. 2.

The rise and fall of blood pressure afecting substances during the course of autolysis of fresh yeast is shown in fig. 3. Thus; pure culture of Sac. cereviciae is subjected to autolysis in a thermostat regurated at 40°C., eight samples are taken in the course of a handred hours, and are dried in a vacuum desiccator under special care. Every sample is divided in two parts, one as it is (broken lines), another is extracted with alcohol to remove the soluble matters (full lines). It is noticeable that the effects of autolysates of different stages are very complicated while corresponding alcohol insoluble parts decrease the blood pressure of rabbit throughout the stages.

It suggests intermediate formation of blood pressure increasing substance, with unstable nature, during the autolysis of yeast.

Since it is impossible to avoid a certain degree of autolysis in preparing dried yeast in bulk, the obscurity of the effects of dried yeast on blood pressure become complehensive.

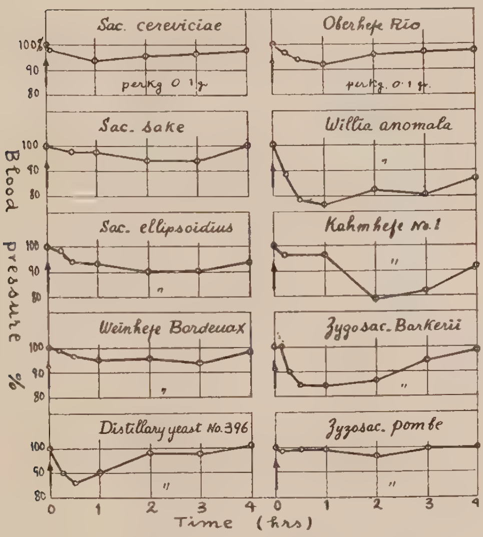

Fig. 2 Effects of alcohol insoluble part of dried yeasts of different species on the blood pressure of rabbit.
per kg 0.1 g per os.

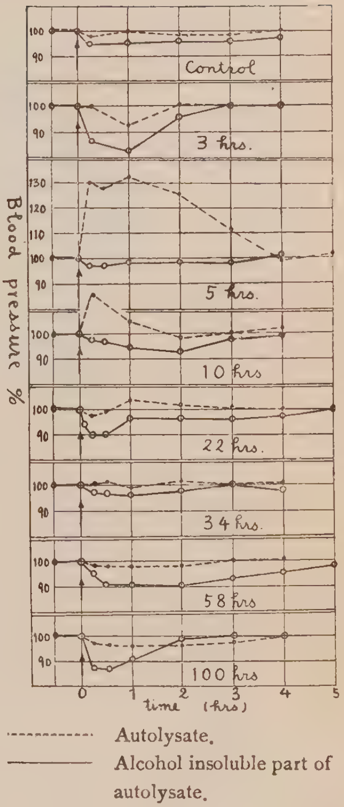

Fig. 3 Blood pressure affecting substances in the course of autolysis of fresh yeast. Rabbit; per kg 0.1 g per os.

Studies on Blood Pressure Decreasing Substances.

Part. 2. Yeast nucleic acid.

By

Masao Toki and Tohkichi Miyoshi

(Received April 17, 1933.)

Sodium nucleate.

When an aqueous solution of sodium nucleate is administrated to a rabbit

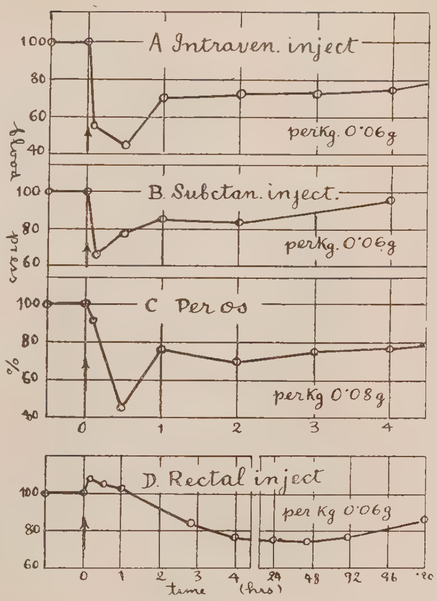

Fig. 1 Effects of Na-nucleate on the blood pressure of rabbit.

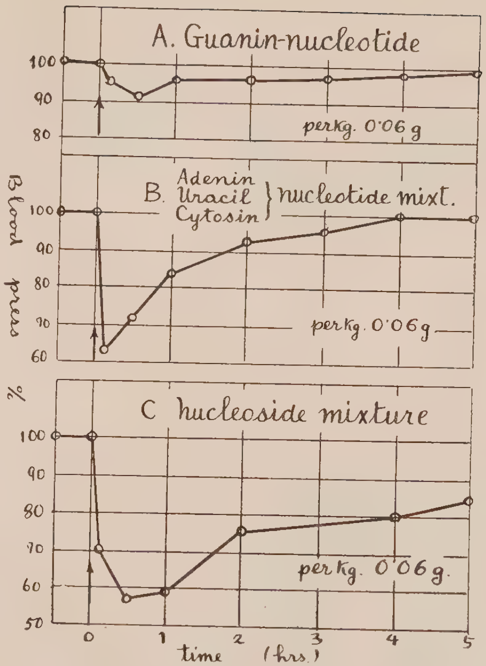

Fig. 2 Effects of intermediate hydrolytic products of nucleic acid on the blood pressure of rabbit. Intraven. inject.

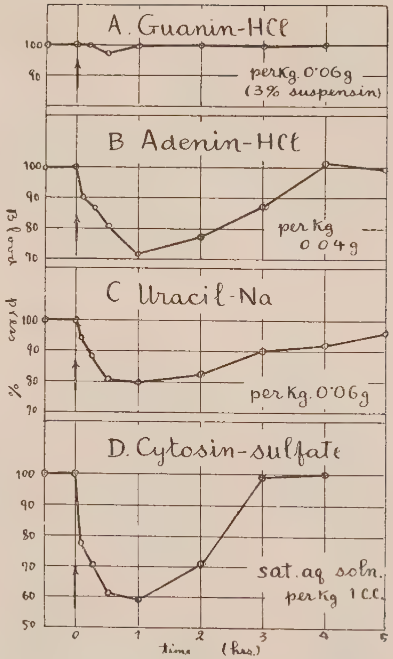

Fig. 2 Effects of intermediate hydrolytic products of nucleic acid on the blood pressure of rabbit. Intraven. inject.

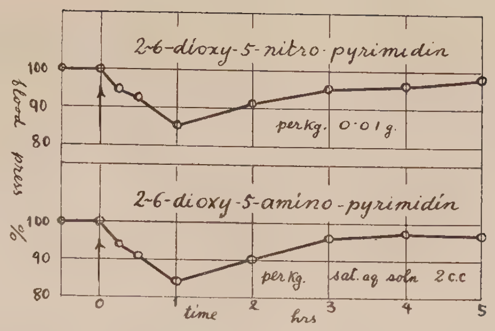

Fig. 4 Effects of synthetic pyrimidins on the blood pressure of rabbit. Intraven. inject.

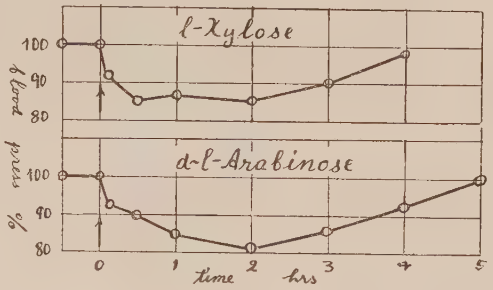

Fig. 5 Effects of aldopentoses on the blood pressure of rabbit. per kg 0.2 g ; intraven. inject.

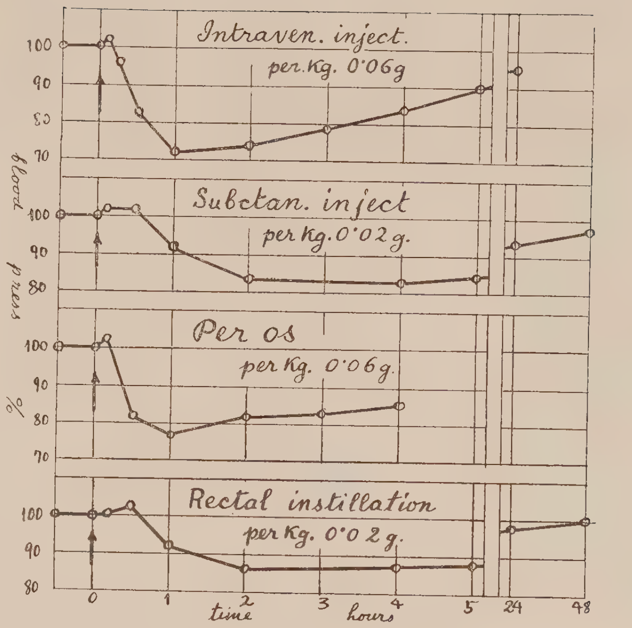

Fig. 6 Effects of Na-phosphate on the blood pressure of rabbit.

by one of the means of intraven. inject., subctan. inject, per os or rectal instillation, the blood pressure is remarkablly depressed in every case (fig. 1).

Hydrolytic products of nucleic acid.

(1) Intermediate hydrolytic products.

Both nucleotides and nucleosides decrease the blood pressure of rabbit when injected intravenously (fig. 2).

(2) Final hydrolytic products.

a) Bases.

Adenin, uracil and cytosin have strong blood pressure decreasing activity while that of guanin is obscure (fig. 3).

According to the fact that two of the synthetic pyrimidins, 2,6–dioxy–5–nitro–pyrimidin and 2,6–dioxy–amino–pyrimidin, also decrease the blood pressure, it is likely to suppose that the blood pressure decreasing activity is an attribute of pyrimidins (fig. 4).

b) Pentose.

Unexpectedly, when an aqueous solution of l–xylose is injected intravenously the blood pressure of the rabbit is decreased and same result is observed in the case of d. l–arabinose (fig. 5). The similarities of intensity of the effect and the shape of the curves suggest aldopentoses, including d–ribose, generally have blood pressure decreasing activity.

c) Phosphoric acid.

When an aqueous solution of sodium phosphate is administrated to a rabbit by one of the means of intraven. inject., subctan. inject., per os or rectal instillation a remarkable depression of blood pressure is observed in every case (fig. 6).

Regarding to the biochemical importance an independent presice report concerning only to the phosphoric compounds will be contributed before long.

Sterilising Action of Acids. III Report.

Sterilising action of saturated monobasic fatty acids on putrifactive bacteria, Bac. typhosus and V. cholerae.

(The Second Report.)

By

Sogo Tetsumoto

(Received April 19, 1933.)

I reported in the preceding edition(1) concerning the sterilising action of saturated monobasic fatty acids (from formic to capric) on putrifactive bacteria, Bac. typhosus and Vib. cholerae. Now I studied the effect of monobasic higher fatty acids, pH, anions and undissociated molecules conserning the sterilising action on microorganisms.

(1) Reagents, rational formulae and pH.

These are noted in next table.

Table 1.

Number of C atom	Acids	Rational formulae	Molecular Weight	pH of saturated solution
C_{11}	Undecylic	$CH_3(CH_2)_9 \cdot CO_2H$	186.231	6.0
C_{12}	Lauric	$CH_3(CH_2)_{10} \cdot CO_2H$	200.250	6.2
C_{14}	Myristic	$CH_3(CH_2)_{12} \cdot CO_2H$	228.294	6.2
C_{16}	Palmitic	$CH_3(CH_2)_{14} \cdot CO_2H$	256.336	6.2
C_{18}	Stearic	$CH_3(CH_2)_{16} \cdot CO_2H$	284.378	6.2

Each acids are soluble extraordinally small quantity in water. Accordingly I made saturated aqueous solutions at 20°C.……saturated solution of undecylic acid and lauric acid $\doteqdot N/100,000$. myristic, palmitic and stearic acid $< N/100,000$. pH of each acid is determined by Itano's electric quinhydrone method. I could not get acids % C_{13}, C_{15} and C_{17}, so I could not study these acids.

(2) Species of microorganisms and Performance of the experiment.

About these, I took the same process as I reported in the preceding edition(1), (2).

(3) Sterilising action of saturated monobasic higher fatty acids.

Results are shown in the following table.

Table 2. Sterilising action of saturated aqueous solution of monobasic higher fatty acids. (at 20°C).

Acids	pH	Surviving period																													
		Staph. c. pyogen.								Prot. vulgar., H.								Bac. typhosus								Vib. cholerae					
									d		h	d							h	d								m	h		
		2	3	4	8	9	11	12	13	18	24	2	3	4	5	7	8	24	36	3	5	6	8	9	10	5	10	15	2	3	6
Undecylic	6.0	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−
Lauric	6.2	+	+	−	−	−	−	−	−	+	+	±	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	−	−	−	−
Myristic	〃	+	+	+	+	−	−	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−	+	+	+	+	−	−
Palmitic	〃	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	−
Stearic	〃	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	±
Control		+	+	+	+	−	−	−	−	+	+	+	+	+	±	−	−	+	+	+	+	±	−	−	−	+	+	+	+	+	+

+ …… alive − perished ± sometimes alive and sometimes perished

m …… minute h …… hour d …… day

Namely concerning to the sterilising action, undecylic acid is stronger than any acid from C_{12} to C_{18} on saturated aqueous solution at 20°C.

Lauric acid (C_{12}) is weaker than undecylic acid. Acids of myristic to stearic have no sterilising action. And then acids of palmitic (C_{16}) and stearic (C_{18}) give good condition to alive to microorganisms comparing to the control.

(4) Sterilising action at the same pH of saturated monobasic higher fatty acids.

To see the relation between the sterilising action of each acid and pH, I made aqueous solution of pH 6.2 with each acid.

The results are as shown in table 3.

Table 3. Sterilising action at the same pH of saturated monobasic higher fatty acids.

pH 6.2	Surviving period																							
	Staph. c. pyogen.						Prot. vulgar. H						Bac. typhosus						Vib. cholerae					
	3	4	8	9	12	d 13	h 36	d 2	4	5	7	8	2	3	6	8	9	d 10	10	15	m 90	h 2	3	6
Undecylic acid	±	−	−	−	−	−	+	−	−	−	−	−	±	−	−	−	−	−	±	−	−	−	−	−
Lauric 〃	+	−	−	−	−	−	+	±	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−
Myristic 〃	+	+	+	−	−	−	+	+	+	+	−	−	+	+	+	−	−	−	+	+	+	+	−	−
Palmitic 〃	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−
Stearic 〃	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−
Control	+	+	+	−	−	−	+	+	+	±	−	−	+	+	±	−	−	−	+	+	+	+	+	+

To make the solution of pH 6.2 with undecylic acid (pH 6.0), water of 2/3 volume must be added to the saturated undecylic acid solution.

Accordingly the molecular concentration of undecylic acid solution becomes diluent. Owing to this fact, the sterilising power of undecylic acid solution on each microoganisms becomes weaker.

Notwithstanding, even if on the same pH, the sterilising power of undecylic acid (C_{11}) is the strongest of all. Next to this, is lauric acid (C_{12}). Acids of palmitic (C_{16}) to stearic (C_{18}) have no sterilising power. But on Vib. cholerae each acids have weak sterilising power.

By these facts we know that the sterilising action of saturated monobasic higher fatty acids concern little about their pH.

(6) Sterilising action of anions of saturated higher fatty acids.

To ascertain the sterilising action of anions of monobasic higher fatty acids, I made saturated solution of Na, K, Ca and NH_4 salts having the same anions of acids. Results of Na salts and K salts are nearly the same, so I denote here the results of Na salt only. Results are as shown in Table 4.

Table 4. Sterilising action of salts. (I) Na salts.

Na-salts	Surviving period																								
	Staph. c. pyogen.							Prot. vulgar. H						Bac. typhosus							Vib. cholerae				
	3	4	5	8	9	13	d 14	2	3	4	5	7	d 8	2	3	6	7	8	9	d 10	10	20	m 30	h 3	6
undecylate	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−
laurate	+	+	−	−	−	−	−	+	±	−	−	−	−	+	+	−	−	−	−	−	+	+	−	−	−
myristate	+	+	+	+	+	+	−	+	+	+	+	−	−	+	+	+	+	+	−	−	+	+	+	+	−
palmitate	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	−
stearate	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	−
Control	+	+	+	+	−	−	−	+	+	+	±	−	−	+	+	±	−	−	−	−	+	+	+	+	+

(II) Ca salts.

Ca salts	Surviving period																											
	Staph. c. pyogen.							Prot. vulgar. H								Bac. typhosus							Vib. cholerae					
	3	4	5	8	9	11	d 12	h 36	d 2	3	4	5	6	7	8	2	3	5	6	8	9	d 10	10	20	m 30	h 2	3	6
undecylate	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	±	−	−	−	−	−
laurate	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	−	−	−
myristate	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−	+	+	+	+	−	−	−	+	+	+	+	+	−
palmitate	+	+	+	+	+	±	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−
stearate	+	+	+	+	+	±	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−
Control	+	+	+	+	−	−	−	+	+	+	+	±	−	−	−	+	+	+	±	−	−	−	+	+	+	+	+	+

(III) NH_4 salts.

NH_4 salts	Surviving period																											
	Staph. c. pyogen.							Prot. vulgar. H							Bac, typhosus							Vib. cholerae						
	3	4	5	8	9	13	d 14	2	3	4	5	7	8	d 9	2	3	4	6	7	10	d 11	15	20	30	m 45	h 3	6	9
undecylate	+	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−
laurate	+	+	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	−	−	−	−	+	+	±	−	−	−	−
myristate	+	+	+	+	+	−	−	+	+	+	+	+	−	−	+	+	+	+	+	−	−	+	+	+	+	+	−	−
palmitate	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−
stearate	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−
Control	+	+	+	+	−	−	−	+	+	+	±	−	−	−	+	+	+	±	−	−	−	+	+	+	+	+	+	+

From the results of Table 4 we find the following facts.

Anions of saturated monobasic higher fatty acids have weak sterilising action from caprylic (C_8) to lauric (C_{12}). But anions of myristic (C_{14}) to stearic (C_{18}) have no sterilising action respectively. But on Vib, cholerae anions of each acids have weak sterilising action.

The results of Na salts, and NH_4 salts are nearly the same. But if we compare in detail, we know that on NH_4 salts microorganisms survive longest and on Ca salts they perish shortest.

(7) Sterilising action of undissociated melecules.

If we compare the results shown in Table 1 and Table 3, we know that the stearilising power of undecylic acid (C_{11}) and lauric acid (C_{12}) is stronger than their salts respectively. And that their pH has almost no sterilising power. By above facts it is found that the sterilising action of undecylic acid and lauric acid is due to the undissociated molecule besides their anions.

Undissociated molecules of myristic (C_{14}) to Stearic (C_{18}) have no sterilising power. But on Vib. cholerae all these acids have weak sterilising power.

I express my profound thanks to Dr. Y. Tohyama and Dr. S. Kojima for their kind advice on this experiment.

Summaries.

I studied the sterilising action of saturated monobasic higher fatty acids from undecylic (C_{11}) to stearic (C_{18}) on putrifactive bacteria, Bac. typhosus and Vib. cholerae.

These acids are soluble extraordinally small quantity to water. So that I studied on their saturated aqueous solution at 20°C.

Results are as follows

(1) On the saturated watery solution of acids at 20°C, undecylic (C_{11}) to stearic (C_{18}), the sterilising action of undecylic acid is the strongest, lauric acid is the next. Acids of myristic (C_{14}) to stearic (C_{18}) have no sterilising power. These acids give rather good conditions to the microorganisms to alive comparing to control.

But for Vib. cholerae, all these acids show weak sterilising action.

(2) On the sterilising action of these acids, pH concerns little.

(3) Anions of acids C_{11} and C_{12} have weak sterilising power. But anions of acids C_{14}, C_{16} and C_{18}, have no sterilising power.

(4) Among salts of Na, K, Ca and NH_4, NH_4 salts are weakest of all concerning to the sterilisation.

(5) On the sterilising action of undecylic acid and lauric acid, undissociated molecules have also remarkable effect.

References.

(1) S. Tetsumoto: Bul. of the Agr. Chem. S. of Japan, Vol. 8, (1932).
(2) S. Tetsumoto: Bul. of the Agr. Chem. S. of Japan, Vol. 9, (1933).

Studies on the Germination of Seeds. Part III.

Organic Bases of the Seeds, and Seedlings of Soy-Bean Germinated in a Dark Room.

By

Shûiku SASAKI

(From the Biochemical Laboratory, Department of Agriculture, Kyûshû Imperial University.)

(Received April 21, 1933.)

Several organic bases contained in the seeds and seedlings of the soybean were already detected by E. Schulze(1) and K. Yoshimura(2). Their investigation were not, however, exhaustive, and the results seemed different on many points. In view of this, I deemed it not only desirable, but also high interesting to make further research on this subject and to determine definitely, if possible, what variation of bases in the seed may occur during germination, and has done so. The results achieved are briefly reviewed in this paper.

A. Seeds.

(1) Specimen.

The kind of specimen used was the *white autumn seeds* produced in Korea. The results of the general analysis are shown as follows.

Water	11.2 %
Crude fat	16.6
Total nitrogen	7.03
Allbuminous nitrogen	6.48
Non-albuminous nitrogen	0.55
A. Nitrogen precipitated by lead acetate	0.16
B. Nitrogen precipitated by phoschotungstic acid after removing the nitrogen A.	0.24
C. Remaining nitrogen	0.15

(2) Seperation of Bases from Other Substances in the Seeds.

5 kg of the seeds were ground, fat and allied substances were extracted off by ether, and the residue was ground again. The fine powder was extracted five times with 15 L of slightly acetated hot water. The extract was clarified with neutral and basic lead acetate and evaporated to 4 L under diminished pressure, and the bases were precipitated by phosphotungstic and sulphuric acids. The Precipitate was decomposed by baryta, and the excess of baryta was removed by CO_2 gas.

(3) Precipitate by Silver Nitrate (Purine Bases).

The solution of bases was neutralized with nitric acid and precipitated by silver nitrate. The precipitate was treated with hydrochloric acid, and then subjected to the usual method. Two kinds of hydrochloride were resulted, namely, adenine and guanine hydrochloride, and these were separated by means of fractional crystallization. The adenine and the guanine were identified as their chloraurates, picrates, and by the colour reactions.

(4) Precipitate by Silver Nitrate and Baryta (Arginine Fraction).

The filtrate from the precipitate by silver nitrate was treated with more silver nitrate and an excessive quantity of baryta. The precipitate was decomposed by sulphuric and hydrochloric acids, and the free bases were obtained by the usual method.

(a) Histidine :– The solution of bases was saturated with CO_2 gas, and mercuric chloride was added to it. The precipitate was decomposed by H_2S. After removing mercuric sulfide and clarifying it with tannic acid, crystals of histidine dichloride were obtained by the usual method. This chloride was verified by its chlorine content and colour reactions, and it was identified, also, as its picrate, picrolonate, and mono-chloride.

(b) Arginine :– From the filtrate of the precipitate by mercuric chloride, mercury was removed as sulphide, and arginine was obtained as frabianate. It was identified as its nitrate and copper nitrate.

(5) Filtrate of the Precipitate by Silver Nitrate and Baryta (Lysine Fraction).

From the filtrate of the precipitate of the arginine fraction, silver and barium were removed, and the hydrochloride of bases was obtained by the usual method. It was evaporated to dryness and treated with methyl alcohol to remove a large amount of potassium chloride. The solution of methyl alcohol was evaporated to dryness once more and divided into the following two parts by means of cold anhydrous ethyl alcohol.

(a) Choline :– The soluble part in ethyl alcohol was treated with alcoholic solution of mercuric chloride. The precipitate was filtered and decomposed by H_2S, and hygroscopic crystals of choline chloride were obatined by drying. It was identified as its chloraurate and chlorplatinate.

(b) Trigonelline :– The insoluble part in ethyl alcohol contained trigonelline. It was identified as its normal and basic chloraurate, chlorplatinate, and picrate. In this part, the presence of another base was revealed by chloraurate, but on account of the small quantity of it available, further identification was not performed.

B. Seedlings.

(1) Specimen.

In this experiment two kinds of soy-beans were used, namely, *white autumn* *seeds* produced in Korea and *yellow summer seeds* produced in Kagoshima, but the procedures of analysis of the two were exactly same.

Well-selected seeds were sown in a dark room. After 12 days the young plants, having stems and roots of from 12 to 18 cm, were plucked out. There were almost no decayed plants, and the specimens were selected with strict care. The results of the genaral analysis are shown as follows.

Water	87 %
Dry matter	13
In dry matter	
Crude fat	10.5
Total nitrogen	7.53
Albuminous nitrogen	4.26
Non-albuminous nitrogen	3.27
A. Nitrogen precipitated by lead acetate	0.40
B. Nitrogen precipitated by phosphotungstic acid after removing the nitrogen A.	0.96
C. Remaining nitrogen	1.91

(2) Separation of Bases from Other Substances in the Seedlings.

The seedlings, plucked out, selected and washed, were ground at once and extracted with slightly acetated hot water. The following process employed with two cases (3 and 4) were exactly the same as those stated in the preceding paragraphs and are, therefore, not repeated here.

(3) Precipitate by Silver Nitrate (Purine Bases).

(4) Precipitate by Silver Nitrate and Baryta (Arginine Fraction).

(5) Filtrate of the precipitate by Silver Nitrate and Baryta (Lysine Fraction).

The bases of this fraction were, as in the case of seeds, made to the hydrochloride, treated with methyl alcohol, and divided into the following two parts by means of cold anhydrous ethyl alcohol.

(a) The insoluble part in cold anhydrous alcohol (Cadaverine). In this part I isolated a large amount of cadaverine and identified it as its chloraurate, chlorplatinate, and picrate.

(b) The soluble part in cold anhydrous alcohol (Cadaverine and choline). The alcoholic solution was clarified with alcoholic solution of mercuric chloride, and separated into cadaverine and choline by means of chloraurate and chlorplatinate, for the chloraurate of cadaverine is more soluble in water than that of choline and the chlorplatinate of choline is more soluble in water than that of cadaverine. These substances were identified as their several salts.

In this experiment I accertained that Stanék's reagent for choline(3) could not be used successfully for isolating choline from the mixture of choline and cadaverine, because cadaverine is also precipitable with this reagent.

C. Summary and conclusion

(1) Adenine, guanine, histidine, arginine, trigonelline, choline, and a small quantity of an unknown base were isolated from soy-bean seeds; and adenine, guanine, histidine, arginine, choline and cadaverine from its seedlings. I obtained these substances quantitatively as far as possibly could. The results of the experiment are shown in the following table setting them side by side with those of Schulze and Yoshimura.

Table 1.

	Schulze		Yoshimura		Sasaki		
	Seeds	Seedlings	Seeds	Seedlings	seeds	Seedlings	
					A**	A**	B**
Adenine	——	——	0.02	present	0.10	0.55	0.42
Guanine	——	——	——	present	0.06	0.12	0.08
Histidine	——	present	present	——	0.08	1.60	2.01
Arginine	0.33	increase*	0.07	——	1.40	3.33	——
Choline	0.61	1.47	0.08	present	1.33	1.40	1.71
Trigonelline	——	——	0.01	——	0.05	present?	——
Betaine	present ?	——	——	present	——	——	——
Cadaverine	——	——	——	——	——	1.22	1.16
An unknown base	——	present ?	——	——	0.16**	——	——

Remarks: –

(1) All figures in the table represent g. of bases isolated from 1 kg of seeds by calculation.

(2) Schulze stated only "increase by germination", but did not gave any figures to the amount of arginine in seedlings (* mark).

(3) The figures in the raw under the A** marks above are taken from the result of the experiment with the *white autumn seeds* produced in Korea, and those in the B** mark obtained from that with the *yellow summer seeds* produced in Kagoshima, Japan.

(4) The figure 0.16*** are represent the amount of chloraurate of an unknown base.

(2) It will be noted that adenine, guanine, arginine and histidine increase in quantity during germination, and markedly so with histidine.

(3) Choline does not so much increase during germination as Schulze set forth.

(4) A little quantity of trigonelline is found in seeds, but the presence of it in seedlings is questionable.

(5) Cadaverine is contained in large quantities in the seedlings. It has not been detected in any higher plant, so far as known.

(6) Stanék's reagent for choline dose not meet the purpose when cadaverine is present.

(7) An unknown base contained in the seed was isolated, but its veri-

fication had not been carried out, because the quantity obtained was so small that it was impossible.

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On the Natural Pigments of Raw Silk Fibre of the Domestic Cocoon. (Part V).

Detection of Violaxanthin in the yellow Cocoon.

By

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The natural yellow pigments of the domestic yellow cocoon were hitherto identified by the author as chiefly constituted of xanthophylls, from which crystalline pigment was isolated and identified as lutein (m. p. 193°). The mother liquor of lutein, however, has yet the tinge of deep orange red and deemed me the presence of another more oxygen containing pigment than lutein. I have examined the presence of violaxanthin ($C_{40}H_{56}O_4$) in this fraction and isolated it in minor quantity in the crystalline state, in major in the resinous substance and identified it as violaxanthin, taking advantage of its color reaction against several reagents, comparing with lutein ($C_{40}H_{56}O_2$) and fucoxanthin ($C_{40}H_{56}O_6$).

Experimental.

Procedure of isolation.

To the 85% methanol mother liquor of lutein (of Chinese No. 7 yellow species) was added petroleum benzin (b. p. 70～80°) and as much water as adjusting the concentration of methanol to 70%, rejecting minor quantity of crystals of lutein thereby formed. Then the upper layer of petroleum benzin containing xanthophylls was separated and again agitated with 70% methanol and again separated the upper. This petroleum benzin solution was washed with water and dehydrated and brought to the chromatographic analysis as Tswett given, taking calcium carbonate as adsorbent. The chromatogram there formed was differentiated into three zones, showing the major part of xanthophyll adsorbed near the top of the adsorbent. This top zone of the chromatogram was taken out and extracted with pure methanol. Methanol solution was again submitted to the fractionation with petroleum benzin and 70% methanol and chromatographic analysis followed as already given. The top zone of the chromatogram was extracted with ether, ether evaporated and the residue was repeatedly recrystallized from pure methanol from which very minute amount of pigment was separated which melts at 185°, leaving the major in the resinous state.

Identification of violaxanthin.

(1) Solvent :- readily soluble in $CHCl_3$, ether, soluble in methanol, ethylalcohol, CS_2, insoluble in petroleum ether.

(2) Absorption spectrum :- three absorption bands were perceived which coincide with those of violaxanthin.

(3) Behavior toward alkali treatment :- showed resistance toward 50% alcoholic potash solution and showed no change or its absorption spectrum which proved to be distinguished from fucoxanthin.

(4) Color reaction :- ether solution of the pigment was treated with following reagents and observed color reaction thereby occurred.

(a) HCl :-

18% HCl.........showed no coloration
19% " ....showed indigo blue ring a little later
20.5% " .... " " " " immediately
22% " .... " " " " momentary
25% " .... " " " " strongly & momentary

(b) Formic acid :- to the crystal was added conc. formic acid, when there developed green color first, then dissolved changing to indigo blue coloration.

(c) Picric acid :- ether solution of picric acid was added which resulted olive color after two minutes, yellowish green after ten minutes, which is very stable even later.

(d) Glacial acetic acid :- yellowish first, indigo blue afterward.

(5) Fractional distribution between 70% methanol and equivalent mixture of petroleum ether and ethyl ether :- a part of the pigment was extracted from the later with the former.

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